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BACTERIAL TOXICITY AND METABOLISM OF THREE HYDRAZINE FUELS.(U)
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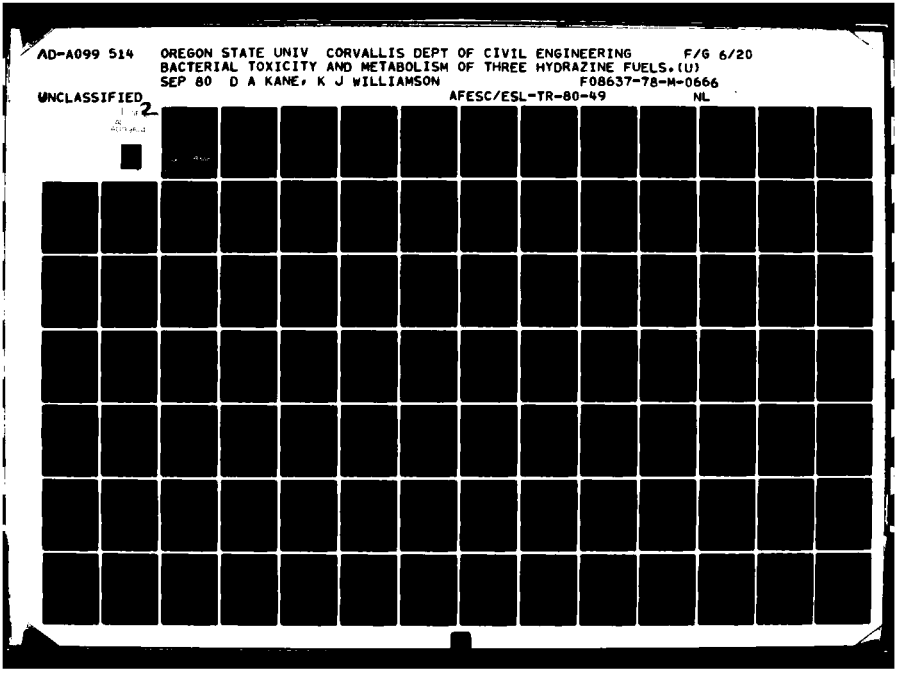

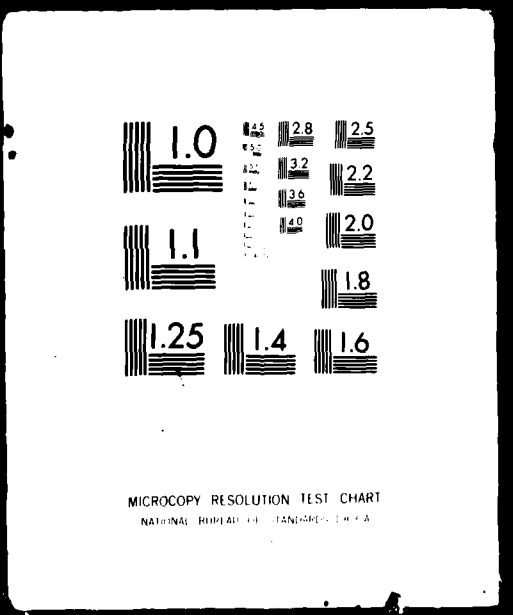

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BACTERIAL TOXICITY AND METABOLISM OF THREE HYDRAZINE FUELS

LEVEL II

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Hydrazine based fuels are used by the Air Force for the Titan II and Minuteman III missiles and the F-16 aircraft and by the Air Force and NASA Space Shuttle Program. These uses represent significant production, transportation, and storage of these fuels, and, as such, a serious threat to the aquatic environment from the potential for accidental release. This research was undertaken to determine the toxicity of hydrazine (H), monomethyl hydrazine (MMH), and unsymmetrical dimethyl hydrazine (UDMH) to four enriched bacterial cultures:

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Nitrobacter, Nitrosomonas - Nitrobacter, anaerobic bacteria, and denitrifying bacteria. In addition, the metabolism of hydrazine by Nitrosomonas - Nitrobacter was examined.

The toxicity studies utilized batch bioassay methods with response measured in terms of substrate metabolism rates. The results showed that hydrazine produced a 50 percent reduction in metabolism rate for Nitrobacter, Nitrosomonas - Nitrobacter, anaerobic bacteria and denitrifying bacteria at concentrations of about 15, 165, 100 and 100 milligram per liter, respectively; monomethyl hydrazine at 15, <1, 75 and 10 milligram per liter, respectively; and UDMH at 1800, 35, 2300, and 12,500 milligram per liter, respectively.

The metabolism study used ^{15}N labeled hydrazine sulphase with high vacuum techniques followed by mass spectographic analysis of the captured gas. Nitrosomonas were found to metabolize hydrazine to nitrogen gas on a short term basis but could not metabolize MMH or UDMH. However, Nitrosomonas were unable to acclimate to long-term dosage of hydrazine.

It was concluded that spills of these three fuels could be expected to seriously disrupt the natural bacterial balance in the aquatic environment. In addition, use of biological waste treatment for detoxification of these three fuels is not recommended.

PREFACE

This report documents research by Lt Col Donald A. Kane performed at Oregon State University as partial fulfillment of the requirements for the award of his Ph.D. This research was performed under Contract No. F08637-78-M0666 with the Civil Engineering Department, Oregon State University, Corvallis, Oregon, during the period 10 January 1978 to 15 August 1979.

This research was accomplished under Program Element 63723F Project 21037W93 and the Project Officer was Lt Col Michael G. MacNaughton.

The report has been reviewed by the Public Affairs Office and is releasable to the National Technical Information Service (NTIS). At NTIS it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

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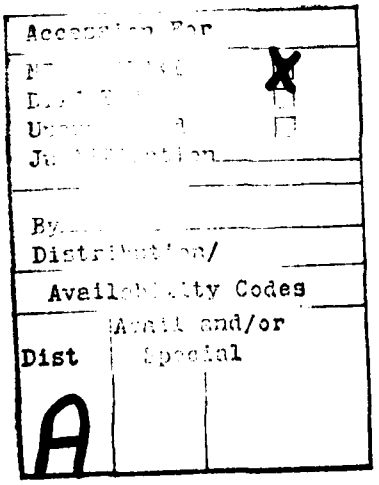

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SECTION I

INTRODUCTION

Hydrazine, monomethyl hydrazine, and unsymmetrical dimethyl hydrazine are liquid missile fuels used extensively by the United States Air Force (USAF) and by the National Aeronautics and Space Administration (NASA). A real need exists to determine the environmental consequences of an accidental spill and the potential rates of biological degradation in natural environments. Much is known about exposure of plants, animals, and humans, but very little is known about toxicity and possible metabolism by bacteria. Bacteria in both an aquatic and a soil environment would include heterotrophic, autotrophic, and fermentative populations. These same types of bacteria would also be present in biological waste treatment facilities. This research was undertaken to determine the toxicity and possible metabolism of these three fuels by important types of bacteria.

SECTION II

HYDRAZINE FUELS

A. THREE HYDRAZINE FUELS

The three hydrazine fuels of hydrazine (H), monomethyl hydrazine (MMH), and unsymmetrical dimethyl hydrazine (UDMH) were selected based upon their present and future use by the United States Air Force (USAF) and by the National Aeronautics and Space Administration (NASA).

B. USE OF HYDRAZINES

Fisher first isolated and characterized simple hydrazine derivatives in 1875 and suggested the name hydrazine for the basic compound, N_2H_4. He also referred to derivatives of the basic compound as substituted hydrazines including MMH and UDMH (Reference 1). From 1875 until shortly before World War II (WWII), these hydrazine compounds remained very much specialty chemicals. The German effort in using hydrazine in their rocket and jet fuel research in WWII brought the production of hydrazine to a large-scale operation. Since then, the aircraft and space industries have maintained a large demand for the hydrazine compounds (Reference 2).

The uses of hydrazine are not limited to rocket fuels. Hydrazine is a powerful reducing agent and is easily oxidizable. As such, it is used as an antioxidant in boiler water, for cut flower preservation, and for photographic developing. Hydrazine is also used as a surface active agent in plasticizer manufacturing (Reference 1).

As of 1978, most hydrazine and MMH bulk production originated in Lake Charles, Louisiana, and was shipped throughout the United States. UDMH

had been manufactured in Maryland until manufacturing problems and health and environment considerations halted production. Figure 1 shows the distribution of these three fuels and the H/UDMH mix throughout the U.S. (Reference 3).

Current average annual movements of the three hydrazine fuels are as follows (Reference 3):

Hydrazine	2.9×10^6 kg
MMH	1.1×10^6 kg
UDMH	3.1×10^6 kg
H/UDMH Mixture	4.4×10^6 kg

NASA has traditionally used hydrazine and MMH as fuel for small thrust engines throughout the Space Program. NASA and the USAF have also used the hydrazines and mixtures as rocket fuel; a mixture of hydrazine and UDMH in equal amounts is used as the fuel in the Titan II missile. Many of the space vehicles use MMH for attitude control systems. With the advent of the Space Shuttle project, increased use of hydrazine and MMH will occur with storage and use on both coasts. Consequently, as seen in Figure 1, an extensive area of the U.S. will witness transportation of these three fuels.

Recently, the F-16 aircraft was introduced into the USAF inventory and further increased the scope of the geographic location and use of hydrazine. The F-16 aircraft uses a 70/30 mixture of hydrazine and water as an emergency power source. Each aircraft will carry about 26 liters (ℓ) of this mixture. Under engine failure, the mixture will automatically be fed into a heated chamber where the hydrazine decomposition product, nitrogen, will drive a turbine and provide about 10 minutes of emergency

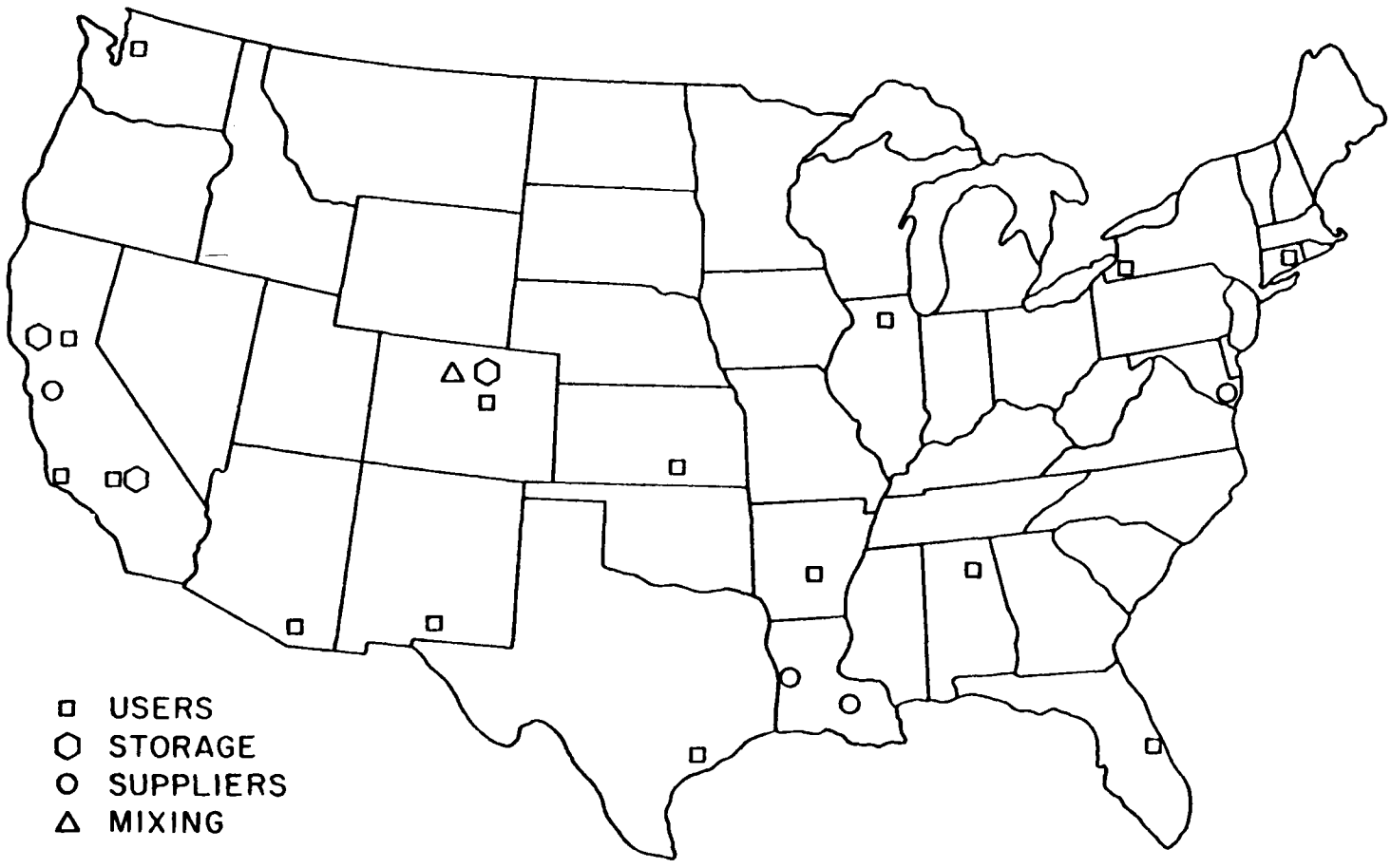

Figure 1. Location of Major Hydrazine Fuel Facilities in United States

electric power. As the F-16 aircraft will also be sold to allied nations, the hydrazine distribution network will extend to other areas of the world.

C. OPPORTUNITY FOR ACCIDENTAL RELEASE TO THE ENVIRONMENT

As principal users of the three hydrazine fuels, NASA and USAF are the agencies most vulnerable to accidental release into the environment. Release can occur in space when the hydrazines are used as thrusters or can occur in the atmosphere when used as a propellant. Future space shuttle missions call for fuel dumping prior to landing to reduce the hazard to the spacecraft and crew. There is also potential for accidental release during storage at the use site where volumes are considerable because current directives call for maintenance of a two-year supply of each fuel (Reference 3).

The greatest potential for accidental release is during transportation of the fuels by rail and truck and during transfer operations. The 55-gallon drum represents the smallest shipping container holding from 740 to 826 kg of fuel, and the largest is the rail car capable of holding from 110,000 to 155,000 kg depending upon type of car employed and type of fuel transported (Reference 3). Accidental release can occur as a result of transportation accidents or during transfer operations, transportation equipment cleaning, and sampling operations.

Based upon the volumes of these fuels manufactured, transported, stored, and used, the potential for accidental release into the environment is considerable. In the event of spills, release into the atmosphere is certain and contamination of the aquatic environment will present a real possibility. The volumes potentially involved and the low concentrations

proven toxic to some aquatic organisms indicate an opportunity for a major environmental disaster. Consequently, this study was undertaken to evaluate the effect and possible mitigation of a hydrazine spill to a small portion of the aquatic environment.

D. PHYSICAL AND CHEMICAL PROPERTIES

Clark (Reference 4) and Audrieth and Ogg (Reference 1) described in detail in the early 1950's the physical and chemical properties of hydrazine. The NASA space effort beginning in the 1960's witnessed a second rebirth in hydrazine-related publications largely by NASA and its contractors. The Olin Corporation did extensive work on its products, hydrazine and MMH, and the FMC Corporation published data on its product, UDMH. This information was then collected, and a compendium was recently published by NASA and its contractor, Florida Institute of Technology (Reference 5). The following information as to the physical and chemical properties of the three hydrazine fuels has been extracted from these three definitive publications.

1. Hydrazine

Hydrazine, like MMH and UDMH, is a clear colorless liquid with a characteristic organic amine odor suggestive of ammonia or fish. It is a liquid at ordinary temperatures and a combustible material. Hydrazine is a highly polar substance and miscible in water, alcohols, ammonia and amines. Thermodynamically, it is unstable and subject to decomposition with attendant energy release. However, it is completely insensitive to shock, friction, or electrical discharge. At normal temperatures, mixtures of hydrazine in

air are flammable between 5 and 100 percent hydrazine by volume. The fire/flash point for hydrazine is about 52°C. Water solutions at concentrations below 40 percent hydrazine will not ignite. Table 1 is a summary of the physical properties of hydrazine, MMH and UDMH (Reference 5).

Early work indicated that the most probable structure for hydrazine was the cis-form with rotation around the nitrogen-nitrogen axis restricted. Additional studies and the fact that hydrazine had a high dipole moment confirmed the cis-form configuration (Reference 1).

2. Monomethyl Hydrazine

Like hydrazine, MMH is insensitive to impact and friction. Spontaneous ignition of MMH can occur either by direct oxidation or when heat evolved from oxidation by atmospheric oxygen is sufficient to ignite rags, cotton cloth, or excelsior that had been soaked with MMH.

3. Unsymmetrical Dimethyl Hydrazine (UDMH)

UDMH is resistant to air oxidation, but will react slowly to form trace products at ambient temperatures. The flash point is lower than hydrazine or MMH although the autoignition temperature is closer to that for hydrazine (see Table 1). Like hydrazine, UDMH will react with carbon dioxide to produce a precipitated salt.

E. DECOMPOSITION PRODUCTS OF HYDRAZINE, MMH AND UDMH

Many investigators have examined the decomposition of the three hydrazine fuels under various conditions. Of particular interest is decomposition of dilute aqueous solutions in the presence of oxygen. The following

TABLE 1. SOME PHYSICAL PROPERTIES OF HYDRAZINE FUELS

Property		N_2H_4	MMH	UDMH
Molecular Weight		32.04	46.08	60.08
Boiling Point (at one atm)	°C	113.5	87.5	63
Freezing Point	°C	2.0	-52.37	-57.2
Liquid Density at 25°C	g/cc	1.0040	.8743	0.784
Critical Temperature	°C	380	312	250
Heat of Vaporization at 25°C	kcal/mole	10.7	9.648	8.37
Heat of Formation (liquid at 25°C)	kcal/mole	+11.999	+13.109	+12.734
Heat of Combustion (liquid at 25°C)	kcal/mole	148.6	311.7	473
Heat Capacity	cal/mole-°C	23.62 @ 25°C	32.17 @ 20°C	39.2 @ 25°C
Flash Point (Tag Open Cup)	°C	52.0	17.2	-15
Autoignition Temperature	°C	270	194.3	250
Flammability Range (Vol. %) in Air		4.7-100	2.5-98	2-95

stoichiometric equations represent the current view of the decomposition of hydrazine, MMH and UDMH:

HYDRAZINE	$2\ N_2H_4 + 0.5\ O_2 \rightarrow N_2(NH_3)_2 + H_2O$	(1)
	$N_2H_4 + O_2 \rightarrow 2\ H_2O + N_2$	(2)
MMH	$(CH_3)N_2H_3 + O_2 \rightarrow 2\ NH_3 + CO_2$	(3)
	$(CH_3)N_2H_3 + 2.5\ O_2 \rightarrow CO_2 + 3\ H_2O + N_2$	(4)
UDMH	$(CH_3)_2N_2H_2 + 2.5\ O_2 \rightarrow 2\ NH_3 + 2\ CO_2 + H_2O$	(5)
	$(CH_3)_2N_2H_2 + 4\ O_2 \rightarrow 2\ CO_2 + 4\ H_2O + N_2$	(6)

Equations (2), (4), and (6) represent the main reaction for the decomposition while Equations (1), (3), and (5) can be viewed as side reactions or intermediate decomposition products which ultimately continue as for the main reactions. Evidence points to N_2, CO_2 and H_2O as the final product in the presence of oxygen of these three fuels. The reactions producing ammonia are supported to some extent by the fact that a typical fishy or ammonia odor is associated with these fuels. It is not clear, however, if the ammonia smell is due to ammonia generated in the aqueous solution or due to the decomposition of the evaporated fuels to ammonia in the mucous membrane of the nose. Many intermediate reactions have been postulated, and their numbers increase with increasing methyl substitutions. For example, UDMH is believed to be partially oxidized to nitrogen gas, water, and formaldehyde dimethyl hydrazine ($2(CH_3)_2NNCH_2$) (Reference 6).

F. TOXICITY OF HYDRAZINE

Each period of renewed interest in hydrazine was accompanied by extensive research into the pharmacology and toxicity of these three hydrazine fuels. Since concern was largely for space and missile launch crews and associated workers, emphasis was placed on human toxicity rather than environmental concerns. In terms of acute toxicity to animals and man, the relative toxicity ranking (least to most toxic) is as follows (Reference 2):

	By Means of:	
Toxicity	Inhalation	Ingestion/Injection
High	MMH	MMH
Intermediate	UDMH	
Low	H	UDMH or H

For hydrazine, acute respiratory exposures to hydrazine for mice and rats show lethal effects at concentrations ranging from 25 to 300 mg/m^3. For dogs, acute respiratory exposure to MMH proved lethal at about 30 ppm and at 110 ppm for UDMH. A wide variation in species tolerances was noted. For all three fuels, the order of decreasing tolerances seem to be hamster, rat, mouse, and dog (Reference 2). There seems to be little indication that the form of the hydrazine used (i.e., free base, salt, or hydrate) plays any significant role in the toxicity of the hydrazine compound.

Since hydrazine and related hydrazine compounds have been in the workplace for decades, exposure standards have been published by various national agencies here and abroad. Prior to the early 1960's, occupational exposure limits on hydrazine were established based primarily on their acute and chronic effects. The three hydrazines of concern produce chronic toxic

effects on the liver, kidneys, and blood. Their acute effects include insult to the nervous system and are manifested by convulsions and other severe signs. The three fuels are also skin and eye irritants (Reference 7).

There are very few cases of accidental human exposure to the hydrazines. German workers during WWII reported eye injuries caused by hydrazine vapor, and in the U.S., dermatitis and eye injury have also been recorded (Reference 7). For MMH and UDMH, the record is almost bare since these chemicals are relatively new arrivals in large quantities. Early acute effect studies indicated that toxic levels of all three hydrazines were in the neighborhood of the odor threshold limits of approximately 5, 3, and 10 ppm for hydrazine, MMH and UDMH, respectively (Reference 7).

In 1962, the hydrazines and the substituted hydrazines were studied for their carcinogenic potential after it was shown that hydrazine sulphate produced neoplasms in mice. Since then, some 19 hydrazine derivatives have proven to be tumor inducers, including hydrazine, MMH, and UDMH (Reference 8). Consequently, all current exposure standards are based on this carcinogenic aspect. The 1979 Threshold Limit Values (TLV) published by the American Conference of Governmental Industrial Hygienists for the three compounds are as follows (Reference 9):

Hydrazine	0.1 ppm
Monomethylhydrazine	0.2 ppm
1,1,-Dimethylhydrazine	0.5 ppm

On the international level, West Germany, Russia, Sweden, and the International Agency for Research on Cancer of the World Health Organization carry the three hydrazines as proven or potential carcinogens (Reference 10).

G. TOXICITY OF HYDRAZINES TO NON-BACTERIAL AQUATIC LIFE

Research on the environmental effects of hydrazines paralleled that of human and animal toxicity studies. However, interest in this area was generated only when the hydrazines were introduced as rocket fuels. In 1959, research conducted with blue gills and flathead minnows showed that both species were sensitive to low concentrations of UDMH, but that different water quality characteristics (pH, oxygen concentration, alkalinity, and hardness) did not impact on toxicity (Reference 11).

Toxic levels of hydrazine and UDMH to aquatic organisms (Daphnia, goldfish, channel catfish, and largemouth bass), rice, and certain plants (endive, alfalfa, pinto beans, and peas) have been reported (Reference 12). In addition to establishing toxic levels, the role of copper as a powerful catalyst in the oxidation and decomposition of the two fuels was documented. When hydrazine and UDMH were applied to soil used for rice seed germination, moderate injury was reported at about 100 ppm for both fuels. When the fuels were mixed in water used for seed germination of non-rice plants, levels of 200 ppm showed no effect. Growth, however, was affected at less than 200 ppm for both fuels. Daphnia were very susceptible to hydrazine with an LD_{50} at 24 hours of 1.2 ppm, and to UDMH with an LD_{50} at 24 hours of 38 ppm. For three fish species, 24-hour LD_{50} values for hydrazine and UDMH were about 4 and 30 ppm, respectively (Reference 12). More recently, additional research on hydrazine toxicity to the three-spine sticklebacks produced a 96-hour LC_{50} at 3 ppm (Reference 13) and a 24-hour LC_{50} ranges for the common guppy of 0.6 to 4.6, 2.6 to 6.7, and 10.1 to 26.5 for hydrazine, MMH and UDMH, respectively. The range variation for each fuel was related to water hardness, with hydrazine in soft water more toxic than in hard water (Reference 14).

Algal bioassay have been conducted which indicates an even lower toxic concentration. In a culture medium equivalent in nutrient status to eutrophic fresh water, an EC_{50} concentration for hydrazine of 0.05 microliters per liter was established based upon a definition of 50 percent reduction in cell growth with hydrazine as compared to controls (Reference 15). This study also produced a safe concentration (SC) of 0.005 microliters per liter under the same conditions. For UDMH, the EC_{50} was about 8.0 microliters per liter and the SC was 0.5 microliters per liter. MMH produced values only slightly less than those for UDMH.

Recently, environmental research involving hydrazine has included the teratogenic effects of these fuels on aquatic organisms. The frog has been used in this research because it lives and breeds in aquatic habitats exhibiting a wide temperature and water quality range. Tests showed that all hydrazine fuels have toxic effects on developing frog embryos and/or larvae. Hydrazine sulphate proved teratogenic at 40 milligrams per liter. MMH was found to be lethal to embryos at greater than 10 milligrams per liter and UDMH lethal to all embryonic stages at greater than 100 milligrams per liter and still highly teratogenic at 10 milligrams per liter (Reference 16).

SECTION III

BACTERIA POPULATIONS OF INTEREST

A. BASIS OF SELECTION

Four bacteria populations were selected for the study of microbial toxicity on the basis of involvement in the nitrogen cycle. The nitrifying bacteria Nitrosomonas and Nitrobacter are two genera that play the major role in nitrogen oxidation. Denitrifying bacteria convert the products of nitrification to nitrogen gas. Anaerobic bacteria involve continuous recycling of nitrogen compounds especially from organic to inorganic forms. These four populations (Nitrosomonas, Nitrobacter, denitrifying bacteria, and anaerobic bacteria) were also selected because of their ubiquitous nature in the aquatic environments and their major roles in waste water treatment processes. The relationship of the bioassay populations, the nitrogen compounds of interest, and the nitrogen oxidation states are outlined in Figure 2.

Of the four groups, Nitrosomonas was later selected for study of the fate of hydrazine in a microbial system.

B. NITROSOMONAS - NITROBACTER

A colony of mixed Nitrosomonas - Nitrobacter was employed even though Nitrosomonas was the bacteria of interest because of an inability to establish a pure culture of Nitrosomonas with our simplified culture procedures. The symbiotic Nitrosomonas - Nitrobacter culture was studied along with a separate Nitrobacter culture to allow conclusions to be drawn about Nitrosomonas by subtraction.

The mixed Nitrosomonas - Nitrobacter population was of special interest

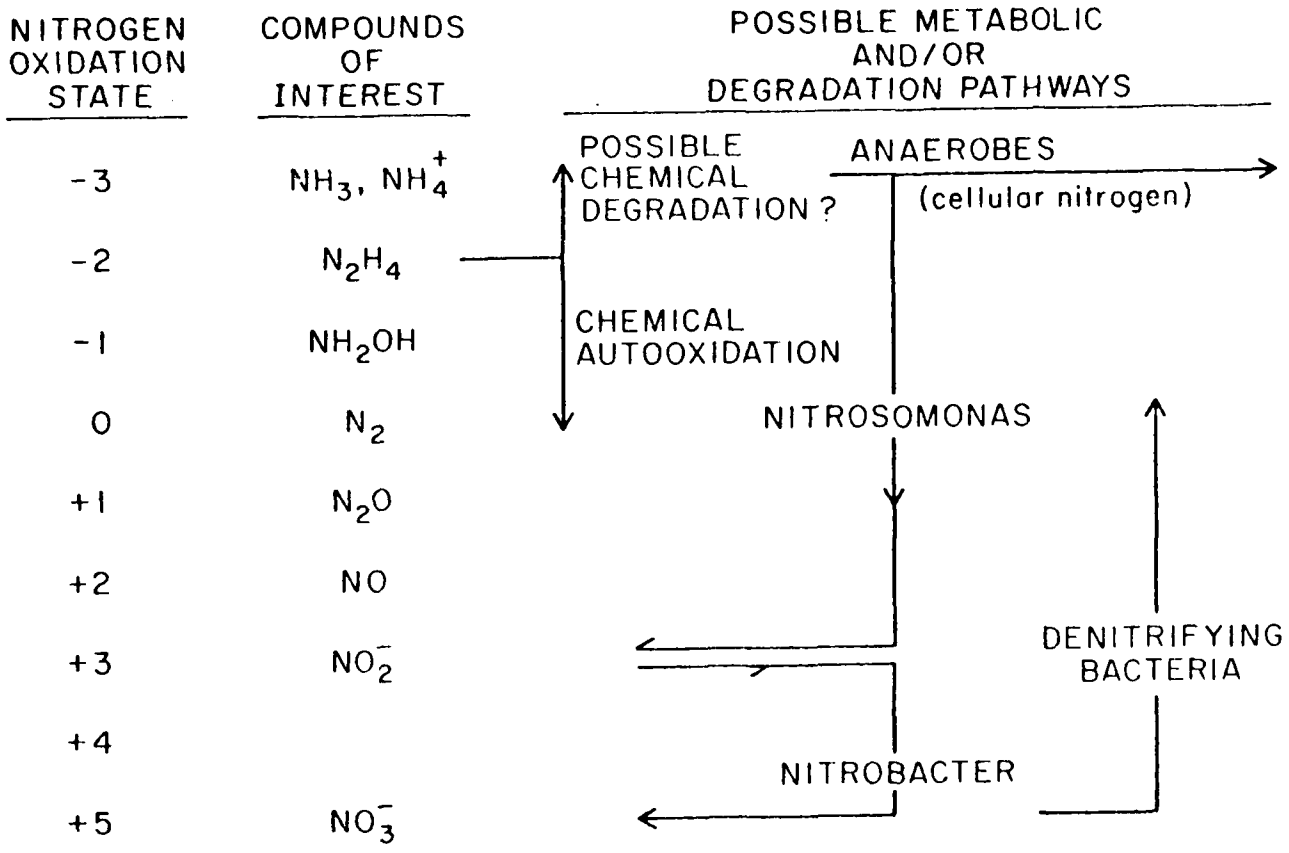

Figure 2. Metabolism Schematic for Various Nitrogen Compounds

because it utilizes a substrate (NH_4^+ - NH_3) at only one level lower than the nitrogen oxidation state of hydrazine and, as such, could conceivably metabolize hydrazine. In addition, Nitrosomonas are known to be more resistant to various toxic agents than Nitrobacter including hydrazine (Reference 17). Thus, Nitrosomonas could possibly degrade sufficient quantities of hydrazine at sub-lethal levels.

Nitrosomonas and Nitrobacter are two autotrophic bacteria found together in soil, sewage, manure, mud, and similar aquatic habitats. Nitrosomonas are obligate autotrophs and strict aerobes which receive their energy from the reaction (Reference 18):

$$NH_4^+ + \frac{3}{2}\,O_2 \rightarrow 2\,H^+ + H_2O + NO_2^- \qquad (7)$$

Nitrobacter are aerobic autotrophs (Reference 29) which receive their energy from the reaction (Reference 30):

$$NO_2^- + \frac{1}{2}\,O_2 \rightarrow NO_3^- \qquad (8)$$

The cell metabolism and growth for both bacterial groups combined can be represented by the following equation if the formula for cell mass of $C_5H_7O_2N$ is accepted (Reference 18):

$$48\,NH_4^+ + 87\,O_2 + 94\,HCO_3^- \rightarrow C_5H_7O_2N + 47\,NO_3^- + 50\,H_2O + 90\,H_2CO_3 \qquad (9)$$

This also reflects the requirement that between 6.0 and 7.4 milligrams of alkalinity are utilized per milligram NH_4^+ oxidized to NO_2^-.

Equation (7) represents the overall oxidation of NH_4^+ to NO_2^-, but this is not a one-step process. Many researchers have shown that this is at least a two-step process (References 19,20,21,22,23,24, and 25). The oxidation state change is from $-3(NH_4^+)$ to $+3(NO_2^-)$. Aleem and Nason (Reference 26) have proposed the following three-step change involving two electron transfers (oxidation states in brackets):

$$\underset{[-3]}{NH_4^+} + \frac{1}{2}\,O_2 \rightarrow \underset{[-1]}{NH_2OH} + H^+ \rightarrow \underset{[+1]}{\text{unknown}} + O_2 \rightarrow \underset{[+3]}{NO_2^-} + 2\,H^+ + H_2O \quad (10)$$

Hydroxyalamine (NH_2OH) has been positively identified as an intermediate at the -1 oxidation state. The possible unknown intermediates at the +1 oxidation have been postulated to be NOH, N_2O, or $H_2N_2O_2$ (Reference 20).

A wide variety of compounds have been reported as toxic to Nitrosomonas. Some compounds were determined to be toxic based on studies where the compound itself was of interest. In other cases, toxicity and/or inhibition by certain compounds were reported where the purpose was to use inhibitors as tools to determine metabolic pathways or biochemical transformations.

Tomlinson, Boon and Trotman (Reference 17) measured the concentrations of 59 chemicals necessary to cause 75 percent inhibition of ammonia oxidation in activated sludge (Table 2). Chemicals commonly used in industry and agriculture and that might be discharged into rivers and sewage treatment facilities were chosen for study. In another study 12 compounds including hydrazine sulphate were tested for toxicity to Nitrosomonas and Nitrobacter (Table 3). No attempt was made to determine the mechanism of inhibition or toxicity of these compounds.

TABLE 2. INHIBITORY EFFECT OF VARIOUS ORGANIC COMPOUNDS ON THE OXIDATION OF AMMONIA BY ACTIVATED SLUDGE

Compound		Inhibition Concentration (mg/l)
Thiourea	$(NH_2)_2CS$	0.076
Thioacetamide	$CH_3.CS.NH_2$	0.53
Thiosemicarbazide	$NH(NH_2).CS.NH_2$	0.18
Methyl isothiocyanate	$CH_3.NCS$	0.8
Allyl isothiocyanate	$CH_2:CH.CH_2.NCS$	1.9
Dithio-oxamide	$NH_2.CS.CS.NH_2$	1.1
Potassium thiocyanate	KCNS	300*
Sodium methyl dithiocarbamate	$CH_3.NH.CS.S\ Na$	0.9
Sodium dimethyl dithiocarbamate	$(CH_3)_2.N.CS.S\ Na$	13.6
Dimethyl ammonium dimethyldithio-carbamate	$(CH_3)_2.N.CS.S.NH_2(CH_3)_2$	19.3
Sodium cyclopenta-methylene-dithiocarbamate	$C_5H_9.NH.CS.S\ Na2H_2O$	23
Piperidinium cyclo-pentamethylene-dithiocarbamate	$C_5H_9.NH.CS.S.NH_2C_5H_{10}$	57
Methyl thiuronium sulphate	$NH_2.C(:NH).S.CH_3\ _2H_2SO_4$	6.5
Benzyl thiuronium chloride	$NH_2.C(:NH).S.CH_2(C_6H_5)\ HCl$	49
Tetramethyl thiuram monosulphide	$(CH_3)_2.N.CS.S.CS.N(CH_3)_2$	16
Tetramethyl thiuram disulphide	$(CH_3)_2.N.CS.S.S.\ CS.N(CH_3)_2$	30
Mercaptobenzothiazole	$C_6H_4.SC(SN):N$	3
Benzothiazole disulphide	$C_{14}H_8N_2S_4$	38
Phenol	$C_6H_5.OH$	5.6
o-cresol	$CH_3.C_6H_4.OH$	12.8
m-cresol	$CH_3.C_6H_4.OH$	11.4
p-cresol	$CH_3.C_6H_4.OH$	16.5
Aniline	$C_6H_5.NH_2$	7.7

TABLE 2. INHIBITORY EFFECT OF VARIOUS ORGANIC COMPOUNDS ON THE OXIDATION OF AMMONIA BY ACTIVATED SLUDGE (CONCLUDED)

Compound		Inhibition Concentration (mg/l)
2-4 dinitrophenol	$C_6H_4(NO_2)_2$	460
Allyl alcohol	$CH_2{:}CH.CH_2OH$	19.5
Allyl chloride	$CH_2{:}CH.CH_2Cl$	180
Di-allyl ether	$(CH_2{:}CH.CH_2)_2O$	100
Sodium cyanide	NaCN	0.65
Dimethyl p-nitroso-aniline	$(CH_3)_2N.C_6H_4.NO$	19
Guanidine carbonate	$(NH_2)_2.C{:}NH\ H_2CO_3$	16.5
Diphenyl guanidine	$(NH.C_6H_5)_2.C{:}NH$	50*
Diguanide	$NH_2C(:NH)NH.C(:NH)NH_2$	50
Dicyandiamide	$NH_2.C(:NH)NH.CN$	250
Skatole	$C_6H_4NHCH{:}CCH_3$	7.0
Strychnine hydro-chloride	$C_{21}H_{22}O_2N_2.HCl.2H_2O$	175
2-chloro-6-trichloromethyl-pyridine	$C_5H_3NCl(CCl_3)$	100
Ethyl urethane	$NH_2.CO.OC_2H_5$	1,780
EDTA	$(COOH.CH_2)_2.N.CH_2\ 2$	
Hydrazine	$NH_2.NH_2$	58
Methylamine hydrochloride	$CH_3.NH_2HCl$	1,550
Trimethylamine	$N(CH_3)_3$	118
Sodium azide	NaN_3	23
Methylene blue	$C_{16}H_{18}N_3SCl.3H_2O$	100*
Carbon disulphide	CS_2	35
Ethanol	C_2H_5OH	2,400
Acetone	$CH_3.CO.CH_3$	2,000
Chloroform	$CHCl_3$	18
8-hydroxyquinoline	$C_9H_6N.OH$	72.5
Streptomycin	$C_{21}H_{39}N_7O_{12}$	400*

*Highest concentration tested, but not effective.

TABLE 3. INHIBITION LEVELS OF VARIOUS COMPOUNDS ON THE ACTIVITY OF NITROSOMONAS AND NITROBACTER IN ACTIVATED SLUDGE

Compound	Inhibition of Nitrosomonas (M)	Inhibition of Nitrobacter (M)
Hydrazine sulphate	2×10^{-2}	1.5×10^{-3}
Sodium azide	3.6×10^{-4}	2.2×10^{-4}
Sodium arsenite	1.3×10^{-2}	5×10^{-2}*
Sodium cyanide	3.5×10^{-5}	5.7×10^{-5}
Sodium cyanate	2.5×10^{-3}**	2.5×10^{-3}
2.4 dinitrophenol	2.5×10^{-3}	2.2×10^{-3}
Dithio-oxamide	1.5×10^{-5}	3.5×10^{-4}
Methylamine	2.3×10^{-2}	5×10^{-2}***
Trimethylamine	2×10^{-3}	4.3×10^{-3}
Potassium chromate	3.5×10^{-3}	2.8×10^{-2}
Potassium chlorate	2×10^{-2}	2×10^{-3}
Nickel sulphate	4×10^{-4}	5×10^{-3}

At maximum concentration tested:

* - Inhibited by 65 percent
** - Inhibited by 40 percent
*** - Inhibited by 50 percent

All other inhibited by 75 percent

C. NITROBACTER

Nitrobacter was selected for the toxicity study for several reasons. First, Nitrobacter, like the other cultures, is involved in the nitrification-denitrification processes to which the hydrazine fuels might be related. Second, information about Nitrobacter along with the Nitrosomonas - Nitrobacter colony would allow conclusions to be drawn about Nitrosomonas.

Unlike Nitrosomonas, there is little reason to believe that Nitrobacter can metabolize any of the hydrazine fuels because hydrazine is believed to be oxidized from the -2 oxidation state to the 0 oxidation state of nitrogen gas, whereas Nitrobacter oxidizes nitrite to nitrate from the +3 to the +5 oxidation state of nitrogen. Nitrobacter, however, was selected because of its uniqueness as a bioassay tool to assess toxicity.

Nitrobacter have been used as a bioassay organism by Williamson (Reference 28) to take advantage of four features characteristic of this bacteria:

1. Ubiquitous nature in the aquatic environment.
2. Simple quantification of removal rate by monitoring nitrite depletion.
3. Slow growth rate which offers the use of batch-fed tests with minimal incorporation of substrate into cellular material.
4. High sensitivity to most toxicants as compared to other heterotrophic organisms.

There seems to be little disagreement that Nitrobacter obtains its energy by oxidizing nitrite (NO_2^-) to nitrate (NO_3^-) in a single two-electron transfer step. No intermediate steps or intermediates have been seriously postulated. Cellular carbon is obtained from incorporation of CO_2 by means of the Calvin Cycle.

Other than the effort by Williamson, there has been very little reported in the literature concerning toxicity of various compounds solely to Nitrobacter. Most studies have dealt with the nitrifying bacteria Nitrosomonas - Nitrobacter as a single entity or as a component pair in activated sludge. Consequently, the toxicity data contained in Table 2 also apply to Nitrobacter.

The electron transport system of Nitrobacter has been extensively studied by several researchers (References 30,31,32, and 33). The purpose of most of this research was to determine the mechanism of nitrite oxidation by Nitrobacter, to examine the cytochrome system, or to examine some aspect of the use of the nitrite oxidation energy in CO_2 fixation. Consequently, most of the compounds reported as toxic or inhibitory to Nitrobacter were selected because of their proven inhibitory characteristics. Butt and Lee (Reference 33) showed that nitrite oxidation was inhibited by carbon monoxide and cyanide. Lees and Simpson (Reference 31) found that cyanate, chlorite, chlorate, bromate, iodate, fluoracetate, and nitrourea inhibited nitrite oxidation.

D. DENITRIFYING BACTERIA

The biological process involving the conversion of nitrate nitrogen to nitrogen gas by means of the intermediates nitrite and nitrous oxide is referred to as denitrification. A relative broad range of facultative bacteria can accomplish this process including Pseudomonas, Micrococcus, Archromobacter and Bacillus (Reference 34). The nitrate serves as an electron acceptor, and organic matter serves as electron donor. Thus, the organic matter is used for energy and for synthesis. In the absence of ammonia nitrogen, a small quantity of nitrate is reduced to ammonia for cell synthesis nitrogen (Reference 18).

The stoichiometric relationships in a simplified two-step process can be seen in the following equations using methanol as the organic (Reference 18):

Nitrate to Nitrite

$$NO_3^- + 1/3\ CH_3OH \rightarrow NO_2^- + 1/3\ H_2O + 1/3\ H_2CO_3 \qquad (11)$$

Nitrite to Nitrogen Gas

$$NO_2^- + 1/2\ CH_3OH + 1/2\ H_2CO_3 \rightarrow 1/2\ N_2 + HCO_3^- + H_2O \qquad (12)$$

Overall - Nitrate to Nitrogen Gas

$$NO_3^- + \frac{5}{6}\ CH_3OH + \frac{1}{6}\ H_2CO_3 \rightarrow 1/2\ N_2 + \frac{4}{3}\ H_2O + HCO_3^- \qquad (13)$$

Synthesis

$$\frac{14}{3}\ CH_3OH + NO_3^- + \frac{4}{3}\ H_2CO_3 \rightarrow C_5H_7O_2N + \frac{20}{3}\ H_2O + HCO_3^- \qquad (14)$$

Because oxygen is favored over nitrate as the electron acceptor and because most anoxic environments contain oxygen as well as nitrite and nitrate, nitrite reduction and deoxygenation must be considered when determining the organic requirements. Again using methanol as the organic substrate, the methanol requirement for nitrate reduction, nitrite reduction, and deoxygenation can be expressed as (Reference 18):

$$C_m = 2.47\ NO_3^- - N + 1.53\ NO_2^- - N + 0.87\ DO \qquad (15)$$

where C_m = required methanol concentration, milligrams per liter,

$NO_3^- - N$ = nitrate concentration removed, milligrams per liter,

$NO_2^- - N$ = nitrite concentration removed, milligrams per liter, and

DO = dissolved oxygen removed, milligrams per liter.

The literature on denitrifying bacteria is divided into two aspects. One is the study of the mechanisms of denitrification and a search for intermediates or intermediate pathways. Various inhibitors or toxicants were used in these studies. The other aspect reported in the literature involves laboratory, pilot-sized or full-scale development and testing of nitrification/denitrification schemes. These research efforts yielded data on the effect of temperature, organic loading solids content, and retention times.

Stensel, et al, (Reference 34) reported that while nitrite was an intermediate, no significant buildup of nitrite occurred in a continuous feed laboratory scale denitrification unit. They also reported that there was little change in the rate of denitrification between 20° and 30°C and that the organic material was the growth limiting substrate.

Many researchers have searched for alternate reaction sequences in the reduction pathway from nitrate to nitrite and finally to nitrogen. The reduction of nitrate to nitrite involves a 2 electron transfer from the +5 nitrogen state of NO_3^- to the +3 nitrogen state of NO_2^-. The next reduction step is from the +3 nitrogen state of NO_2^- to the 0 state of nitrogen gas and has generated extensive research. The consensus is that nitrous oxide, N_2O, at the +1 nitrogen state is an intermediate step in the nitrite reduction phase. Early research determined that nitrous oxide was indeed an intermediate compound in nitrite reduction (Reference 35). Sidransky, et al (Reference 36) used azide, acetylene, and cyanide as specific inhibitors and concluded that the pathway from NO_2^- to N_2 included N_2O and that no bypasses of N_2O existed. Using ^{15}N tracers studies, St. John and Hollocher (Reference 37) were able to reach the same conclusions.

In relation to toxic or inhibitory compounds, little is reported about

specific toxicants based upon industrial experience or applied research. Most research has employed known inhibitors including 2,4 - denitrophenol; carbonyl cyanide phenylhydrazine; 3,5 - denitrobenzoate; and zephiran chloride. These inhibitors were used by Walter (Reference 38) concerning the uncoupling of oxidative phosphorylation which disrupted one or more redox reactions in the electron transport system.

E. ANAEROBIC BACTERIA

Extremely diverse and complicated biochemical processes occur in anaerobic environments. These environments can be found in the bottom sediments of lakes and ponds, in anaerobic digestors in sewage treatment facilities, and in pockets of anaerobic soils. The breakdown of complex organics into synthesized cellular material and as a source of cellular energy can be viewed as a three-step process. First, there is the enzymatic hydrolysis of complex organics into soluble and less complex organics. Second, these organics are fermented into simple compounds, primarily fatty acids. The third step is the fermentation of these acids into methane and carbon dioxide. This three-stage process is the rule, but other pathways do exist.

The bacteria performing this complex metabolic process represent a wide range of facultative and anaerobic populations. The more complex the organic waste to be metabolized, the more complex the bacteria groups involved. The term "anaerobic bacteria" is generally applied to the entire population. "Acid formers" is the name used to describe the bacteria found in the second stage of the process (fatty acid formation), and "methane formers" is the term used for the substrate specific obligate anaerobic bacteria fermenting the fatty acids to methane and carbon dioxide (Reference 39).

During this process, many compounds are hydrolyzed and reduced including nitrogen compounds. The nitrogen reduced to NH_4^+ is available for cell synthesis. Consequently, the anaerobic environment offered an additional opportunity to observe the toxicity of hydrazine fuels with special emphasis on the nitrogen of hydrazine. A mixed anaerobic bacteria population was selected since in an accidental spill of a hydrazine fuel or deliberate release into a sewage treatment facility, the anaerobic population exposed will be a mixed one.

The literature regarding toxicity of various compounds to anaerobic bacteria is extensive. Some studies utilized specific species and others use mixed populations. Toxicity was often measured by a decrease in gas production as compared to controls. For a mixed anaerobic population, decreased gas production can be caused by direct inhibition of the methane formers, acid formers, or both. Inhibition could also be due to a pH depression caused by a stimulation of the acid formers rather than a direct inhibition of the methane formers. Table 4 lists some of the reported inhibitory compounds. However, it should be noted that the mixed chemical and biochemical nature of anaerobic environments will have a major impact on the determination of toxic levels for various chemicals. These effects could be antagonistic or synergistic.

TABLE 4. COMPOUNDS FOUND TO INHIBIT MIXED ANAEROBIC BACTERIA

Compound	Concentration mg/l	Source
Sodium	3,500 - 8,000	McCarty (Reference 40)
Potassium	2,500 - 12,000	McCarty (Reference 40)
Calcium	2,500 - 8,000	McCarty (Reference 40)
Magnesium	1,000 - 3,000	McCarty (Reference 40)
Ammonia	1,500 - 3,000	McCarty (Reference 40)
Sulfide	200	McCarty (Reference 40)
Sodium Oleate	500	McCarty (Reference 40)
Acrolein	20 - 50	Gosh & Conrad (Reference 41)
Formaldehyde	50 - 100	Gosh & Conrad (Reference 41)
Crotonaldehyde	50 - 100	Gosh & Conrad (Reference 41)
Methyl isobutyl ketone	100 - 300	Gosh & Conrad (Reference 41)
2 ethyl-1-hexanal	500 - 1,000	Gosh & Conrad (Reference 41)
diethylamine	300 - 1,000	Gosh & Conrad (Reference 41)
acrolyonitrite	100	Gosh & Conrad (Reference 41)
2-methyl-5-ethylpyridine	100	Gosh & Conrad (Reference 41)
ethylene dichloride	150 - 500	Gosh & Conrad (Reference 41)
ethylacrylate	300 - 600	Gosh & Conrad (Reference 41)
phenol	300 - 1,000	Gosh & Conrad (Reference 41)
Chloroform		
Nickel	10 mg/l	Hayes & Theis (Reference 42)
Copper	40	Hayes & Theis (Reference 42)
Chromium (VI)	110	Hayes & Theis (Reference 42)
Chromium (III)	130	Hayes & Theis (Reference 42)
Lead	340	Hayes & Theis (Reference 42)
Zinc	400	Hayes & Theis (Reference 42)
Cyanide	5 - 15	Parkin (Reference 43)
Chloroform	20 - 40	Parkin (Reference 43)

SECTION IV

PROCEDURAL OUTLINE

A. PURPOSE AND SCOPE

The toxicities of the three hydrazine fuels to the four bacteria populations were determined in the first phase. The investigation was limited to short-term acute toxicity to allow extrapolation of results to actual hydrazine spills.

In the second phase, the fates of the hydrazine compound were examined. In the initial procedure the levels of the various nitrogen compounds were monitored to obtain a nitrogen balance to determine the fate of the hydrazine nitrogen. However, a procedure was developed using ^{15}N labeled hydrazine compounds since precise inventory could not always be obtained with a nitrogen balance approach. The second procedure was to be employed if the conventional nitrogen balance procedure failed.

The final phase involved an examination of the long-term effects of hydrazine on Nitrosomonas. Specifically, tests were developed to determine if the bacteria population could acclimate to low hydrazine concentrations and, perhaps, even metabolize hydrazine. These questions related directly to the long-term environmental effects of a hydrazine spill and to the feasibility of biological treatment to detoxify hydrazine.

B. TOXICITY STUDY PROCEDURES

This section describes the general procedures used in the batch bioassay studies. The analytical techniques are outlined in Section V.

1. Definition of Toxicity: For the Nitrosomonas - Nitrobacter and

Nitrobacter populations, toxicity of the hydrazine compounds was defined in terms of a reduction in the substrate utilization rates. A dose-response curve was developed by plotting hydrazine dose versus percent substrate removal. For the denitrifying and anaerobic bacteria, toxicity was defined in terms of a reduction of gas production. Gas production was measured with a respirometer and a dose-response curve was developed by plotting hydrazine dose versus percent gas production. The bioassay period for the anaerobic bacteria was 7 to 10 days to insure that upsets were not due to environmental changes. For the other three bacteria populations, the bioassay period was from 3 to 7 hours.

2. General Procedures: The Nitrosomonas - Nitrobacter culture was developed on an ammonia feed and the Nitrobacter culture, a nitrite feed. Tap water supplied all micronutrients; phosphorus and alkalinity was supplemented in greater than stoichiometric amounts. The bacteria were harvested from the culture chamber and introduced into 125-milliliter Erlenmeyer flasks containing substrate (NH_4^+ or NO_2^-) and the hydrazine dose. The same environmental conditions were maintained in the culture and in the bioassay flasks to minimize the effects of the transfer. The flasks then were placed on a shaker bath and allowed to equilibrate for 30 minutes. The decrease in substrate concentration was monitored until such time as a definite degradation rate was established.

For the denitrifying bacteria, the colony was established utilizing nitrate as the electron acceptor and methanol as the electron donor. Tap water provided the trace micronutrients; phosphorus and alkalinity were added in greater than stoichiometric quantities. Prior to each bioassay, the respirometer flasks were thoroughly purged with nitrogen to remove oxygen. Production

of nitrogen gas was monitored until a constant nitrogen production rate was established.

For the anaerobic bacteria bioassays 1-liter bottles were filled with 600 milliliters of a 50/50 mixture of anaerobic digester sludge and warm tap water and connected to an anaerobic respirometer. Each digester was fed on a daily fill and draw cycle with 30 milliliters of concentrated waste activated sludge. After a period of stabilization, the hydrazine was introduced and gas production monitored for 7 or more days. A dose-response curve was developed on a basis of combined methane and carbon dioxide production.

3. Culture Procedures: The Nitrosomonas - Nitrobacter and the Nitrobacter populations were cultured as shown in Figure 3. The feed was oxygenated with pure oxygen before flowing into the columns containing lightweight plastic beads. The beads served to distribute the flow evenly down through the column and to trap the bacteria in the void spaces. The Nitrosomonas - Nitrobacter feed consisted of tap water plus 30 milligrams per liter N as $(NH_4)_2SO_4$, 1.0 milligrams per liter P as $NaHPO_4 \cdot H_2O$ and $NaHCO_3$ to provide a minimum of 400 milligrams per liter alkalinity. The Nitrobacter feed consisted of tap water plus 20 milligrams per liter N as $NaNO_2^-$ and 1.0 milligrams P as $NaHPO_4 \cdot H_2O$. No additional alkalinity was required.

Primary digester sludge was obtained from the Corvallis municipal waste treatment facility for each anaerobic bioassay. The feed waste activated sludge was obtained once and stored frozen until used. This assured a uniform COD of 20,500 milligrams per liter for the feed.

The denitrifier colony was cultured as shown in Figure 4 (Reference 34). The feed solution consisted of 324 milligrams per liter methanol, 120 milligrams N per liter sodium nitrate, 70 milligrams per liter potassium dihydrogen

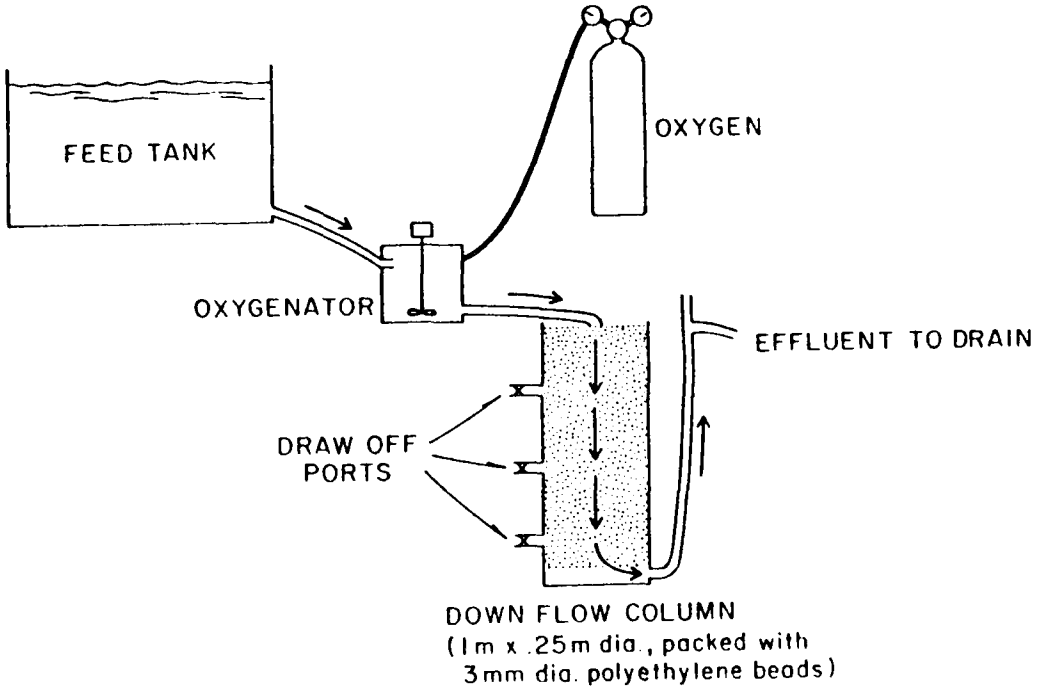

Figure 3. Culture Apparatus for Nitrosomonas - Nitrobacter and Nitrobacter

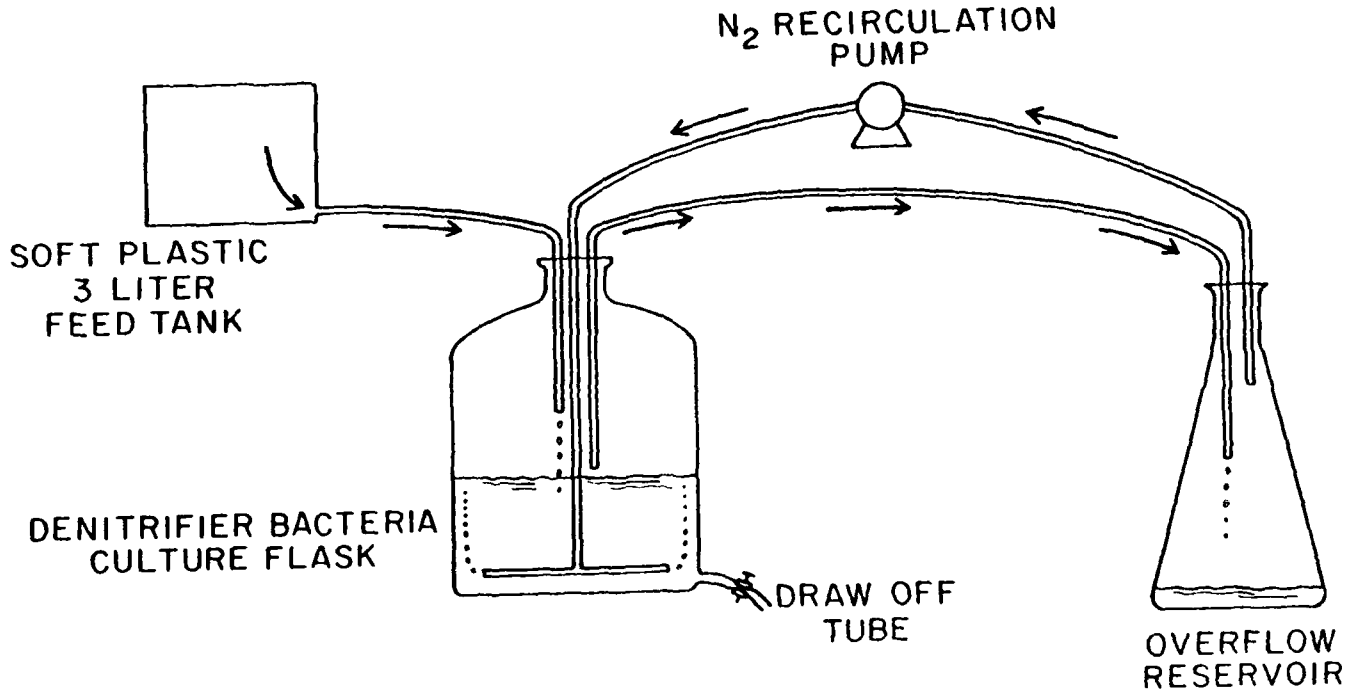

Figure 4. Culture Apparatus for Denitrifying Bacteria

phosphate, and 200 milligrams per liter alkalinity as sodium bicarbonate. It was purged with nitrogen gas for 15 minutes before use and fed from a collapsible container to eliminate the entrance of oxygen.

4. Harvesting Procedures: The Nitrosomonas - Nitrobacter and the Nitrobacter populations were harvested from one or more side sampling ports and concentrated in 1000- or 2000-milliliter separatory funnels. The concentrated bacteria were washed with fresh oxygenated substrate solution. Ten milliliters were transferred to each bioassay flask.

The denitrifiers were harvested by stopping the recirculation pump, allowing the bacteria to settle, and drawing off approximately 75 milliliters of the bacterial concentrate. Three milliliters of the bacteria were transferred to each Gilson respirometer flask and the entire system purged with ultra pure nitrogen.

For the anaerobic bacteria, each bioassay bottle was filled separately with primary sludge and warm tap water (Figure 5). Resumption of normal gas production usually occurred after a 24-hour adjustment period and an additional 10 days were allowed to insure that steady state conditions had been reached. Each reactor was fed daily 30 milliliters of waste activated sludge to give a solids retention time of 20 days. Gas production was recorded every 24 hours.

5. Bioassay Procedures: The two nitrifying population bioassays were accomplished with 100-milliliter liquid volumes in 250-milliliter flasks at a constant pH and temperature. After the addition of the toxicant to each flask the bioassay solution was allowed to equilibrate, and the pH was adjusted with NaOH or H_2SO_4. Harvested bacteria were then introduced into all but two bioassay flasks. For these two flasks,

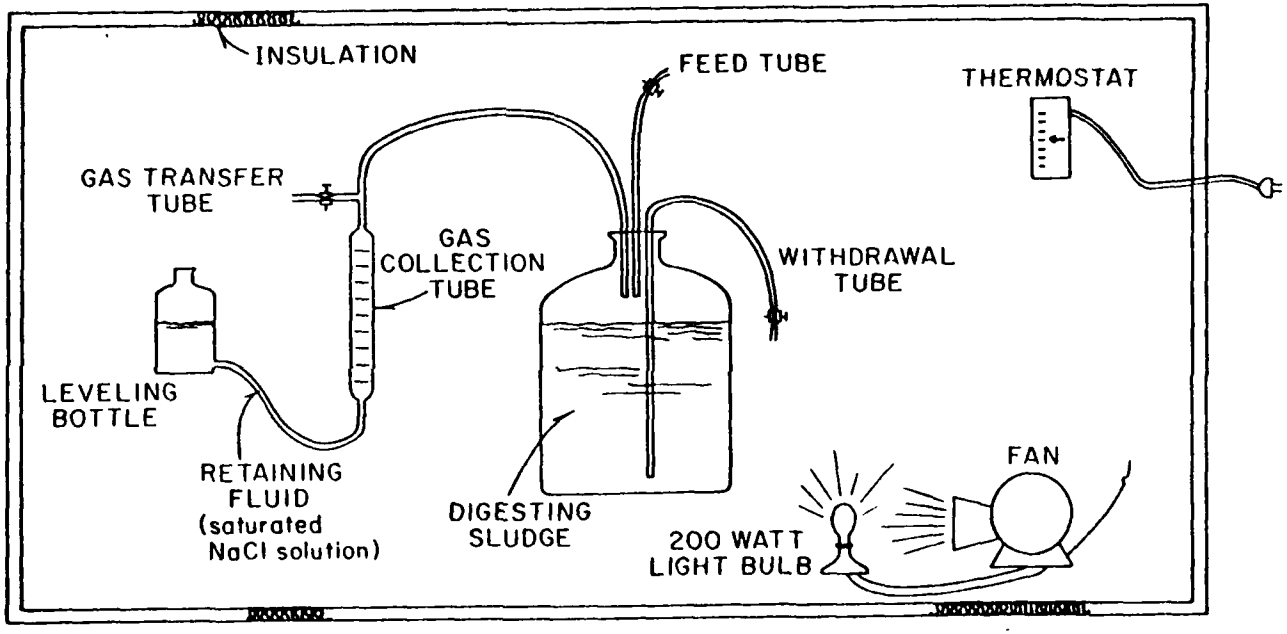

Figure 5. Apparatus for Anaerobic Cultures

bacteria were killed by placing them in a boiling water bath, then rapidly cooled, and introduced.

The Nitrobacter bioassays were sampled initially for nitrite and the toxicant and then for nitrite every 30 minutes. The initial nitrite concentration was about 15 milligrams per liter, and the bioassay was continued until a constant rate of substrate utilization was indicated for controls. At that time, the bioassay was terminated and the solutions filtered to determine total suspended solids (TSS) and volatile suspended solids (VSS). The filtrate was immediately analyzed for nitrite, nitrate, and the toxicant.

For the Nitrosomonas - Nitrobacter bioassays each flask was initially sampled for nitrite and the toxicant, but only the controls were sampled for NH_4^+. Tne controls were sampled for NH_4^+ every hour using the direct Nesslerization method until a constant substrate utilization rate was established. The bioassays containing the hydrazine fuel could not be sampled for NH_4^+ at hour intervals because of interferences by hydrazine in NH_4^+ analysis when using direct Nesslerization (see Section VI for details). The initial NH_4^+ - N concentration was about 15 milligrams per liter. Three initial samples and all final samples were filtered to determine TSS and VSS and analyzed for NH_4^+ and NO_3^- using a specific ion meter and for the toxicant and nitrite by colorimetric methods.

Standard Gilson respirometer procedures were used to assay activity of denitrifying bacteria. To each 15-milliliter flask, 3 milliliters of bacteria were added and 1 milliliter of substrate was placed in the side arm. The substrate solution was identical to the feed solution used to grow the denitrifying bacteria. All flasks were attached to the respirometer, purged with nitrogen, tipped, sealed, and allowed to equilibrate. Nitrogen gas

production was monitored at 30-minute intervals for 5 to 7 hours, and then the flasks were removed and analyzed for TSS, VSS, and toxicants. Initial toxicant concentration was calculated from the known stock concentrations.

SECTION V

ANALYTICAL TECHNIQUES

A. REAGENTS

All reagents used were ACS grade or better. Double glass-distilled water was used for all solutions. Hydrazine sulfate, $H_2NNH_2 \cdot H_2SO_4$ was prepared in a 1000-milligram per liter stock solution and stored without buffering (pH 2). Insignificant degradation was observed under these conditions. Analytical reagent grade monomethyl hydrazine and unsymmetrical dimethyl hydrazine were obtained from the Aldrich Chemical Company and stored at 5°C under a nitrogen blanket. All stock solutions were made fresh daily.

B. FEED SOLUTIONS

All feed solutions were prepared and analyzed to check on dilution techniques and to examine possible interferences. A list of constituents monitored and the method of analysis are in Table 5.

C. HYDRAZINE ANALYSIS

The method of Watt and Chrisp (Reference 44) was employed for hydrazine analysis because of its simplicity, reliability, accuracy, and reproducibility and because only relatively small sample volumes are required. In this test a yellow color develops upon addition of p-dimethylaminobenzaldehyde (DMBA) to solutions of hydrazine under acid conditions. The reagent is prepared by dissolving 10 grams of DMBA in 250 milliliters of 2N H_2SO_4. The original Watt and Chrisp approach used DMBA dissolved in a mixture of ethyl alcohol and hydrochloric acid; however, with no adverse results H_2SO_4 was substituted

TABLE 5. ANALYTICAL METHODS USED FOR CULTURE MONITORING AND BIOASSAY

Compound	Method
NH_4^+	Direct Nesslerization
NH_3	Orion Specific Ion Meter
NO_2^-	Sulfanilic Acid - Naphthylamine Hydrochloride
NO_3^-	(a) Orion Specific Ion Meter
	(b) Brucine
P	Vanadomolybdic Acid
Alkalinity	Acid Titration
pH	Glass Electrode

for the HCl. The DMBA is stable for weeks. The yellow color of the DMBA-Hydrazine solution has a transmittance minimum at 458 nanometers, develops fully in 10 minutes, and is stable for at least two hours (Figure 6). Beer's Law is followed for hydrazine concentrations of up to 6 milligrams per liter using a 1-milliliter sample (Figure 7). Watt and Chrisp (Reference 44) found that the ammonium ion does not interfere with the determination and further tests showed that none of the compounds present in the bioassays interfered with hydrazine determination.

D. MONOMETHYL HYDRAZINE ANALYSIS

The MMH analytical procedures of Reynolds and Thomas (Reference 45) were used. The DMBA is prepared as for hydrazine analysis. A stable yellow color develops with a transmittance minimum at 458 nanometers in the DMBA-MMH solution in 30 minutes and is stable for several hours. Beer's Law is followed for MMH concentrations of up to 60 milligrams per liter using a 1-milliliter sample (Figure 8). When a 3-milliliter sample is used, the lower concentration range can be extended reliably down to 0.5 milligrams per liter. As for hydrazine, no interferences were encountered.

E. UNSYMMETRICAL-DIMETHYL HYDRAZINE ANALYSIS

The method of Pinkerton, et al (Reference 46) was used for UDMH analysis with some minor changes suggested by Appleman (Reference 47). The UDMH sample was diluted in a citric acid-disodium acid phosphate buffer and reacted with trisodium pentacyanoamino-ferrate (TPF). The TPF-UDMH complex gives a fully developed red color with an absorption maximum at 500 nanometers after one hour and is stable for only one to two hours. The TPF reagent itself

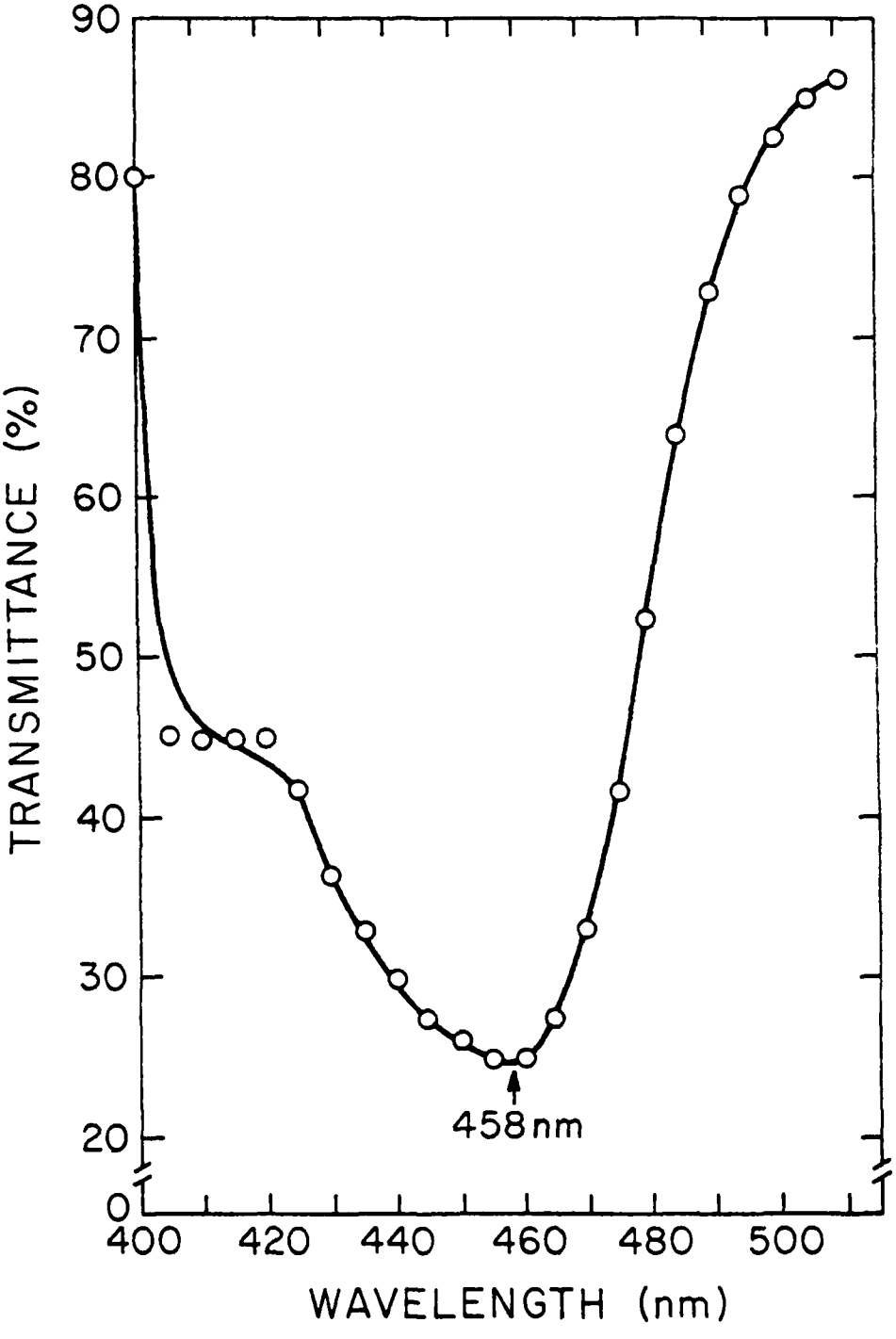

Figure 6. Absorbance Spectra for Hydrazine with p-Dimethylamino Benzaldehyde

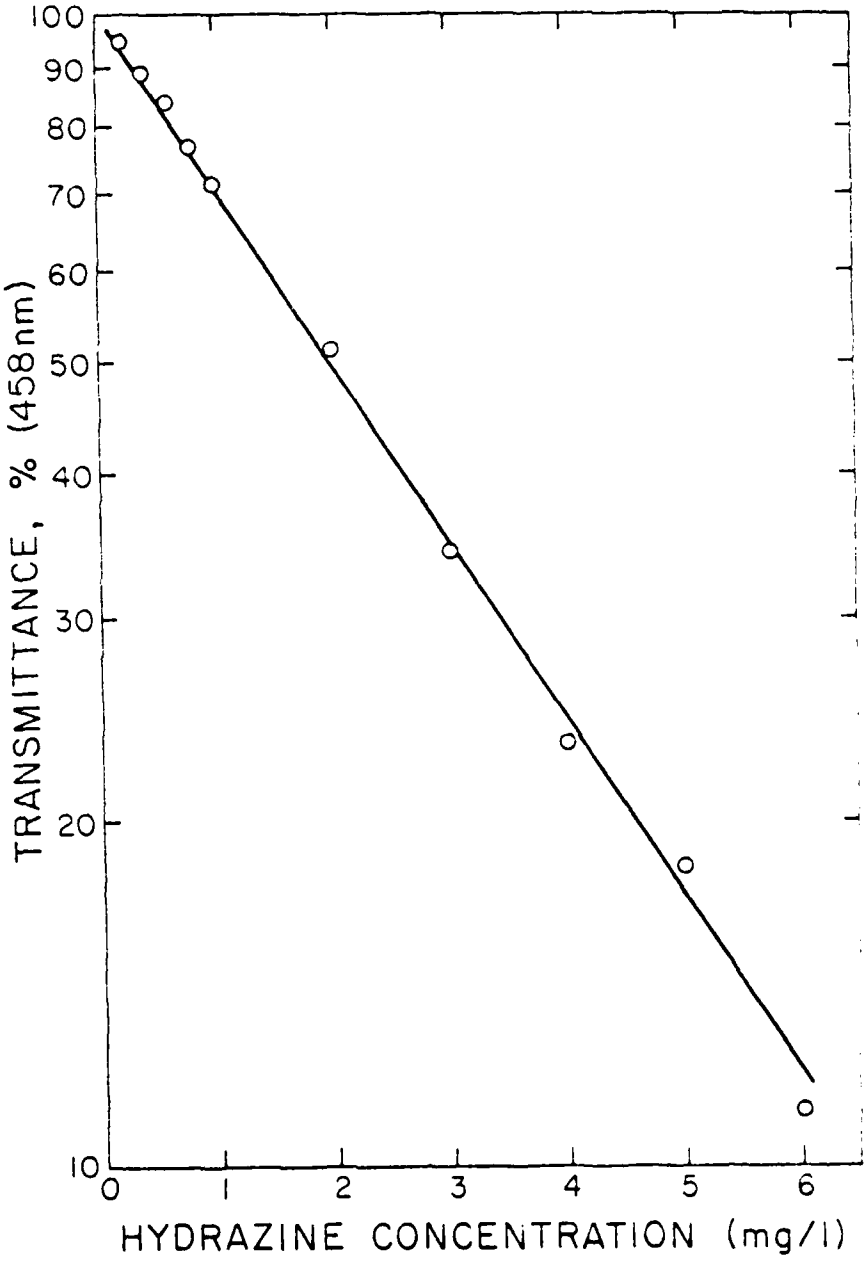

Figure 7. Typical Calibration Curve for Hydrazine

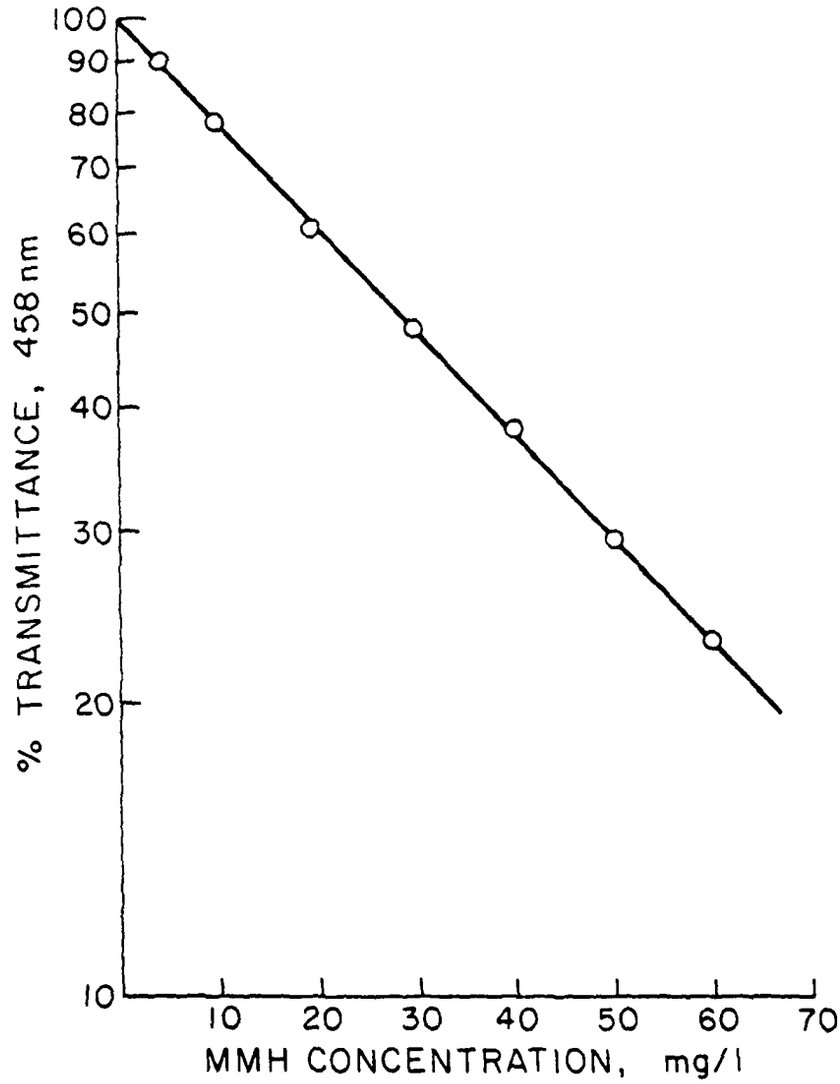

Figure 8. Typical Calibration Curve for MMH

has a yellow color which shows a maximum absorbance at 390 microns, but the TPF-UDMH complex demonstrates maximum absorbance at 500 microns. The respective absorption spectra of the TPF-UDMH complex is shown in Figure 9. The color development followed Beer's Law and was linear up to 50 milligrams per liter with a practical concentration range from 1 to 40 milligrams per liter for a 1-milliliter sample (Figure 10).

F. ^{15}N HIGH VACUUM SYSTEM

A stable isotope tracer method was selected to study the fate of the nitrogen in hydrazine. It was hypothesized that the hydrazine was degraded and/or bacterially metabolized to nitrogen gas. Preliminary tests indicated that a significant amount of hydrazine disappeared in the bioassays and could not be detected as NH_4^+, NO_2^-, or NO_3^-. With the expectation that N_2 was a metabolic product, double labeled ^{15}N-hydrazine was used in a closed system with isolation of gases by high vacuum techniques and subsequent analysis in a mass spectrometer.

The high vacuum technique was similar to that of Dost (Reference 48) (Figure 11) and was designed to be used in a normal ^{14}N-air environment. The gas sample collection device was specifically designed to fit the mass spectrometer in the Department of Agricultural Chemistry, OSU (Figure 12). In one part of the apparatus, the bioassay was conducted in an atmosphere of 20 percent oxygen and 80 percent sulphur hexafluoride (SF_6). The inert, non-reactive SF_6 was used to replace atmospheric nitrogen; the SF_6 is easily condensed in a liquid nitrogen trap (BP-63.8°C). In the second part of the apparatus, the sample is further cleansed of SF_6, residual oxygen is removed, and Freon 14 is injected (mass 69) as an internal standard. Analysis of the

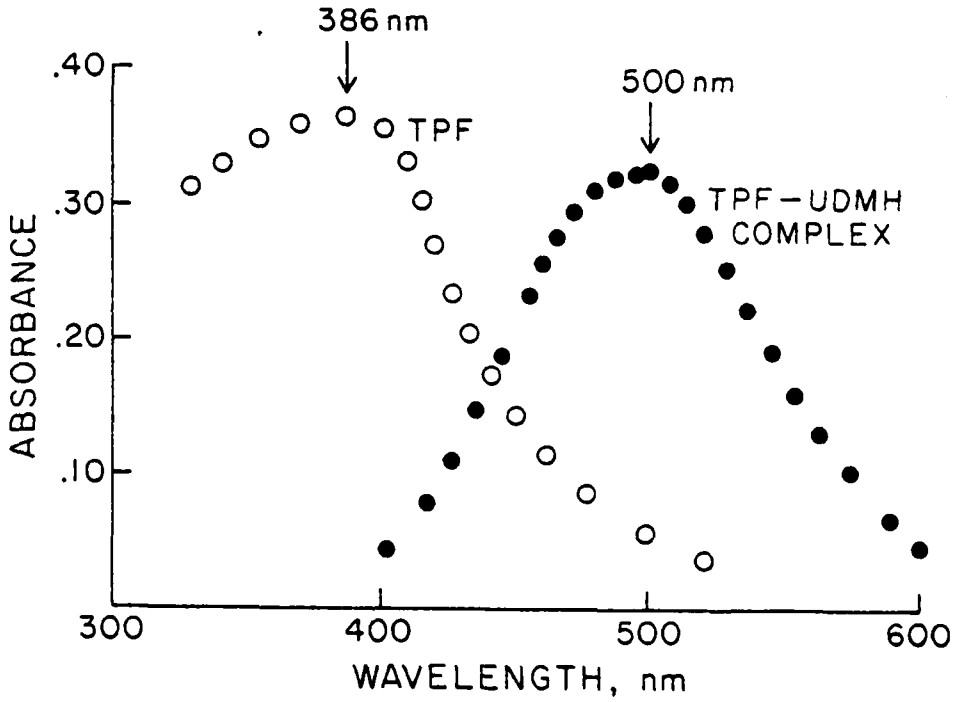

Figure 9. Adsorbance Spectra for TPF and TPF - UDMH Complex

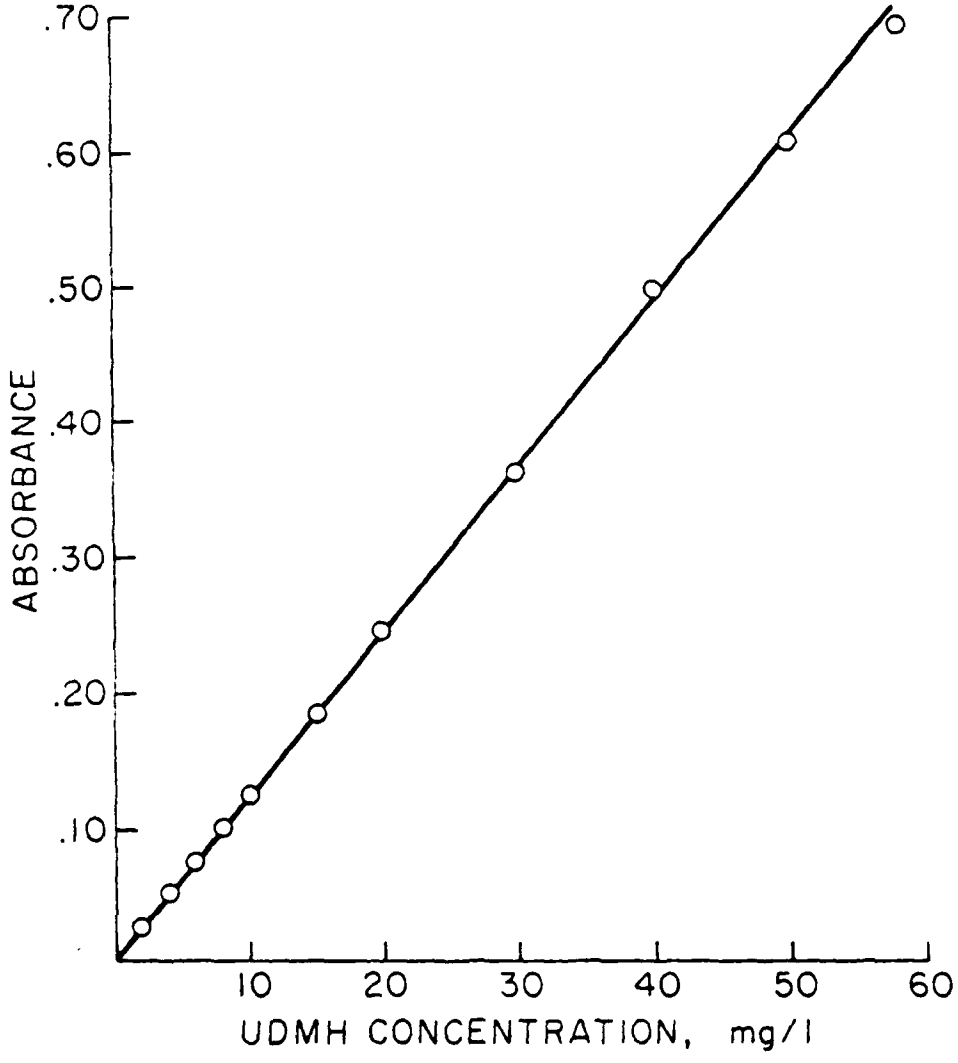

Figure 10. Typical Calibration Curve for UDMH

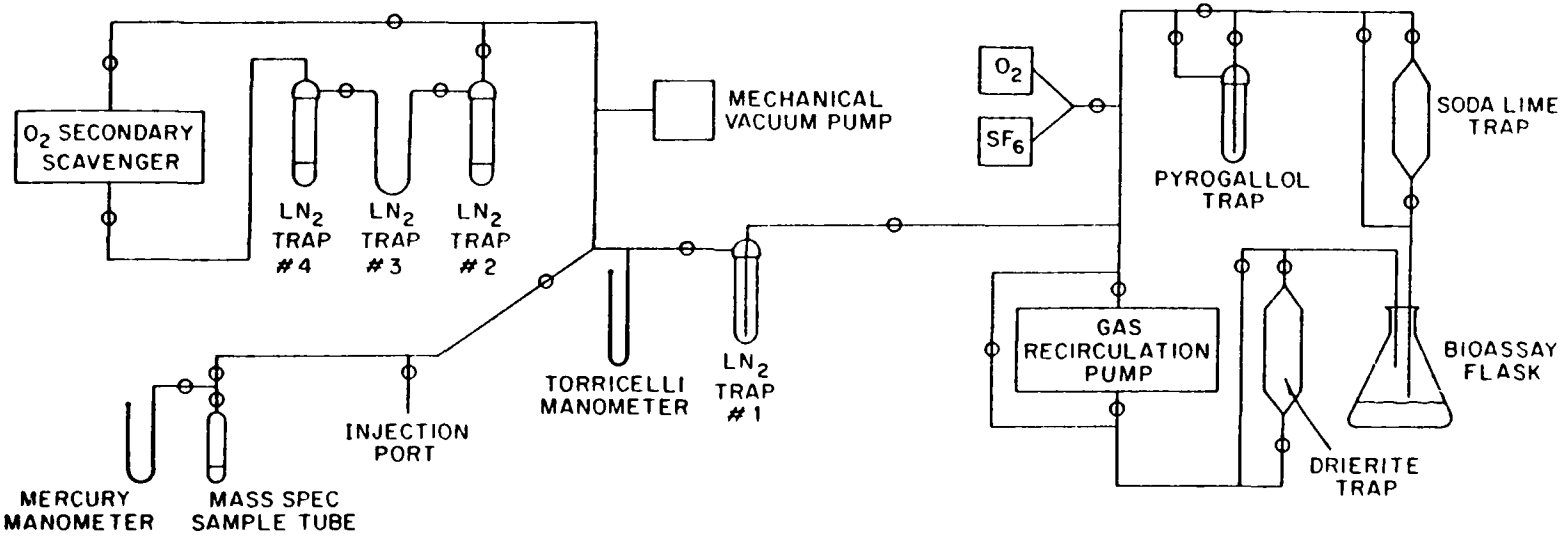

Figure 11. High Vacuum System for Collection of $^{15}N_2$ Gas

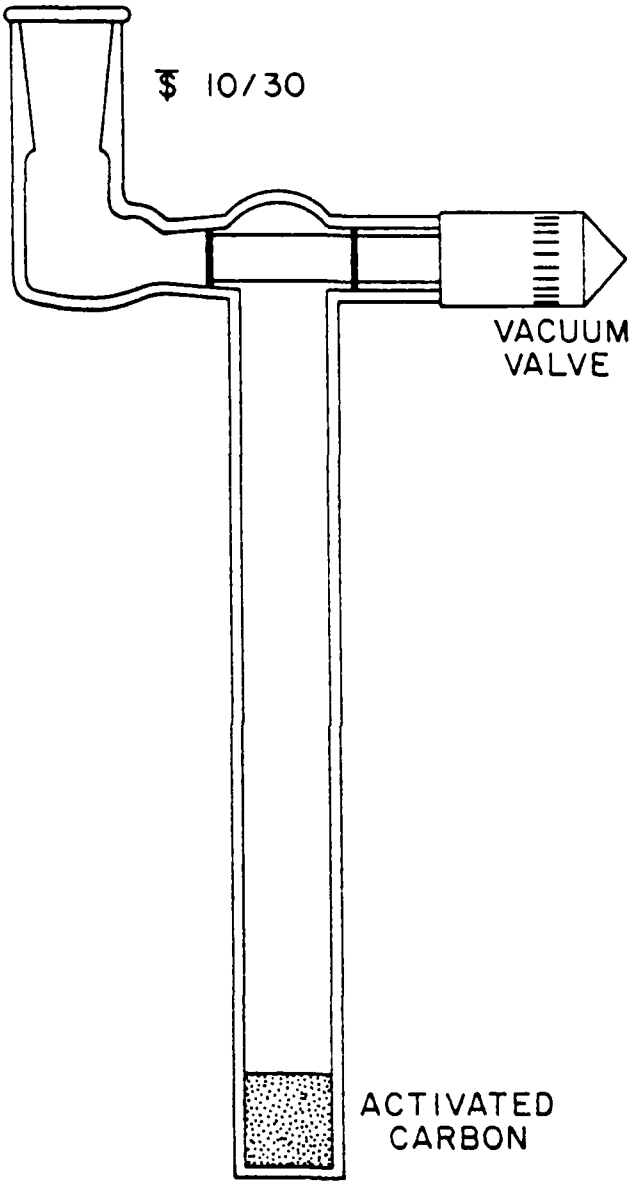

Figure 12. Mass Spectrometer Sample Tube

gases used are listed in Table 6.

The procedures for use of the high vacuum system were:

1. Purge the bioassay side of the system with SF_6 for 20 minutes.
2. Evacuate the entire system to 10^{-1} to 10^{-2} millimeters of mercury and close the valve between the bioassay side and the gas collection side.
3. Open the SF_6 valve and slowly return the bioassay side to atmospheric pressure, open the valve leading to the bioassay flask and purge for 10 minutes, and purge all lines for an additional 10 minutes.
4. Close the valves leading to the pyrogallol trap.
5. Open the O_2 valve and adjust the SF_6-O_2 flow to a 80/20 mix and purge all but the pyrogallol trap for an additional 15 minutes.
6. Connect the 250-milliliter Erlenmeyer flask with the hydrazine/bacteria suspension to the high vacuum system and continue to purge the flask and contents to the atmosphere with the SF_6-O_2 mixture for an additional 15 minutes.
7. Turn off the gas flow and seal the system.
8. Turn on the recirculation pump, run the bioassay, and recirculate gases through the bioassay liquid to insure that oxygen is maintained at saturation levels and that the generated $^{15}N_2$ gas is driven out of the liquid.
9. Terminate the bioassay, record the elapsed time, and open the lines to the pyrogallol trap for 60 to 90 minutes.
10. Check the vacuum level on the vacuum side of the system, turn on the heater for the Oxy-absorbent trap® and set the three liquid nitrogen traps.

TABLE 6. ANALYSIS OF GASES USED IN ^{15}N HIGH VACUUM SYSTEM

OXYGEN, Ultra High Purity	
Moisture	1.7 ppm
Purity	99.99%
SULPHUR HEXAFLUORIDE, SF_6	
Oxygen	0.134%
Nitrogen	0.201%
Moisture	22.5 ppm
Total Hydrocarbons	<10 ppm
Purity	99.0%+
HALOCARBON 14	
Oxygen	0.13%
Nitrogen	0.065%
Moisture	0.0021%
Purity	99.7+%
NITROGEN, Ultra High Purity	
Purity	99.99%

11. Slowly open the valve between the two sides of the system and pull the gases through the liquid nitrogen trap to condense the SF_6, CO_2, NH_3, NO, and N_2O and pass the nitrogen (both $^{14}N_2$ and $^{15}N_2$), oxygen and argon through the main trap and to be adsorbed on the liquid N_2-cooled charcoal in the secondary traps.
12. Pass the trapped gases back and forth through the secondary oxygen trap to remove oxygen not removed in the pyrogallol trap.
13. Close the valve between the two systems and return the bioassay side to atmospheric pressure so as to remove the bioassay flask for analysis and to vent the main SF_6 trap.
14. Inject 1 milliliter of the Freon 14 marker gas through the rubber septum into the system.
15. Inject 5 to 15 milliliters of $^{14}N_2$ through the septum to bring the total sample volume to 10 to 30 milliliters if required.
16. Place the liquid nitrogen trap on the sample tube to recondense gases on to the charcoal in the sample tube.
17. Close the sample tube valve and remove it for subsequent analysis.
18. Re-establish the vacuum on the vacuum side of the system and repeat all procedures with a new sample tube in place, and inject the 1 milliliter of Freon marker, the $^{14}N_2$ gas and 1 milliliter of $^{15}N_2$ gas.

The sample containers remained free of leakage as confirmed by the absence of $^{14}N_2$ and O_2 in the standard containing only $^{15}N_2$ and the Freon marker. Consequently, samples were stored up to 5 to 7 days for convenience. Thick-walled Tygon tubing was used to connect glassware rather than using a rigid all glass system (Reference 48) in order to provide maximum flexibility, to reduce the

susceptibility to breakage, and to facilitate cleaning. All glassware pieces (30°, 60°, 90° bends, Y's, etc.) were connected with 1/2-inch thick (1/2-inch ID, 1-1/2-inch OD) heavy duty Tygon tubing. Each Tygon-glass connection was tightly clamped. The tubing did not collapse or crack under vacuum.

The pyrogallol, Drierite, soda lime, and Oxy-absorbent traps® were emptied and replaced prior to each run. The activated carbon traps in the sample tube were heated two minutes under vacuum to drive off any adsorbed gases. When not in use, the sample collection side of the system was kept under vacuum.

SECTION VI

INTERFERENCE STUDIES

Possible interferences by the hydrazines were a major concern in choosing the analytical procedures. Hydrazine fuels are powerful reducing agents and, as such, interfere with standard colorimetric NH_4^+ and NO_3^- analyses. The hydrazine fuels also were found to interfere with the standard nitrite analysis but not significantly. This interference became significant only when the hydrazine-to-nitrate ratio exceeded about 100 to 1 or the MMH and UDMH ratio exceeded about 1000 to 1. Both of these ratios were much larger than the ratio used in the bioassay tests.

The specific ion electrode methods for NH_4^+ and NO_3^- were evaluated and found free of significant interferences from the hydrazine fuels. However, even though the specific ion methods proved to be acceptable, a penalty was paid in that 5- to 100-milliliter samples were required. Since sampling for these two nitrogen compounds before, during, and after each bioassay would consume almost the entire sample, sampling was limited to only before and after each bioassay.

The specific ion electrode method has a reported accuracy to within ± 5 percent. However, this accuracy could not be sustained at low concentrations of NH_4^+ and NO_3^- (below 3 milligrams per liter). Such low concentrations are typical of the starting NO_3^- concentration and the ending NH_4^+ concentrations for controls. Consequently, a precise nitrogen balance could not be obtained and could not be used to support the hypothesis that hydrazine was degraded to nitrogen gas. Consequently, the nitrogen gas also had to be measured and the ^{15}N labeling technique was chosen.

SECTION VII

CHEMICAL DEGRADATION OF THE HYDRAZINE FUELS

A. CHEMICAL DEGRADATION OF HYDRAZINE

Hydrazine degradation in various solutions including distilled water, tap water, phosphate in tap water, nitrite in tap water, ammonia in tap water, and sterile primary sewage effluent was studied. Hydrazine did not degrade in distilled water and only slightly in tap water. Degradation was minimal in 10 milligrams per liter NH_4^+ -N and 10 milligrams per liter NH_4^+ -N plus 0.2 milligrams per liter P (as KH_2PO_4). However, hydrazine degraded rapidly in tap water containing only 0.2 milligrams per liter phosphate and in the sterile sewage (Figure 13).

Due to rapid degradation in solutions containing phosphate or sewage, further studies were initiated to provide more detailed information. Specifically, degradation effects of sterile and non-sterile solutions were examined. To obtain the sterile sewage and hydrazine concentrations, solutions were autoclaved in 250-milliliter flasks and hydrazine added after autoclaving.

Hydrazine degraded rapidly in both the sterile and non-sterile sewage (Figure 14), with the non-sterile environment producing the most rapid rate. The degradation in the phosphate solutions were slower than in the sewage. No significant effect of hydrazine concentration was noted which suggests a zero-order reaction rate.

Hydrazine was added to sewage before autoclaving in some experiments and it was found that only about 40 percent of the hydrazine remained after autoclaving. Repeated testing showed that most of this loss occurred immediately after introduction of the hydrazine into the sewage. Immediate

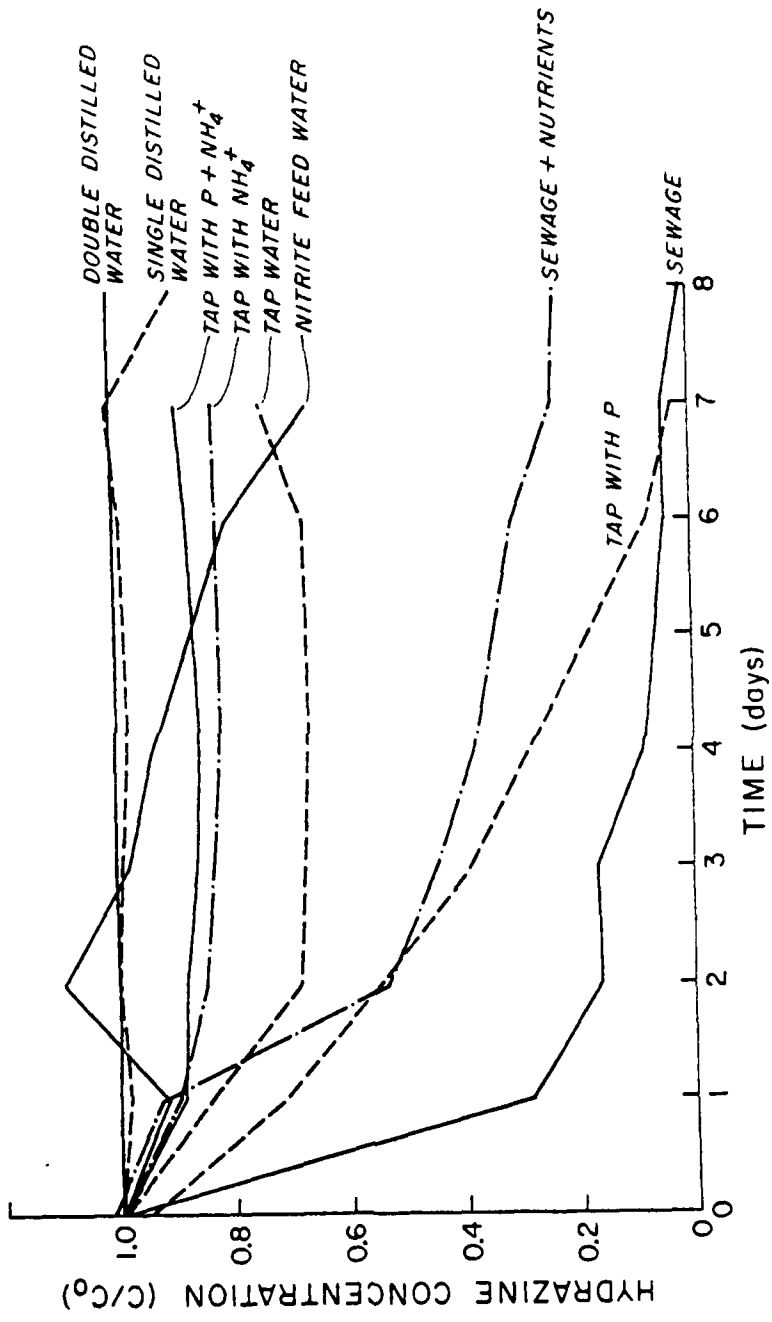

Figure 13. Degradation of Hydrazine in Various Solutions (A)

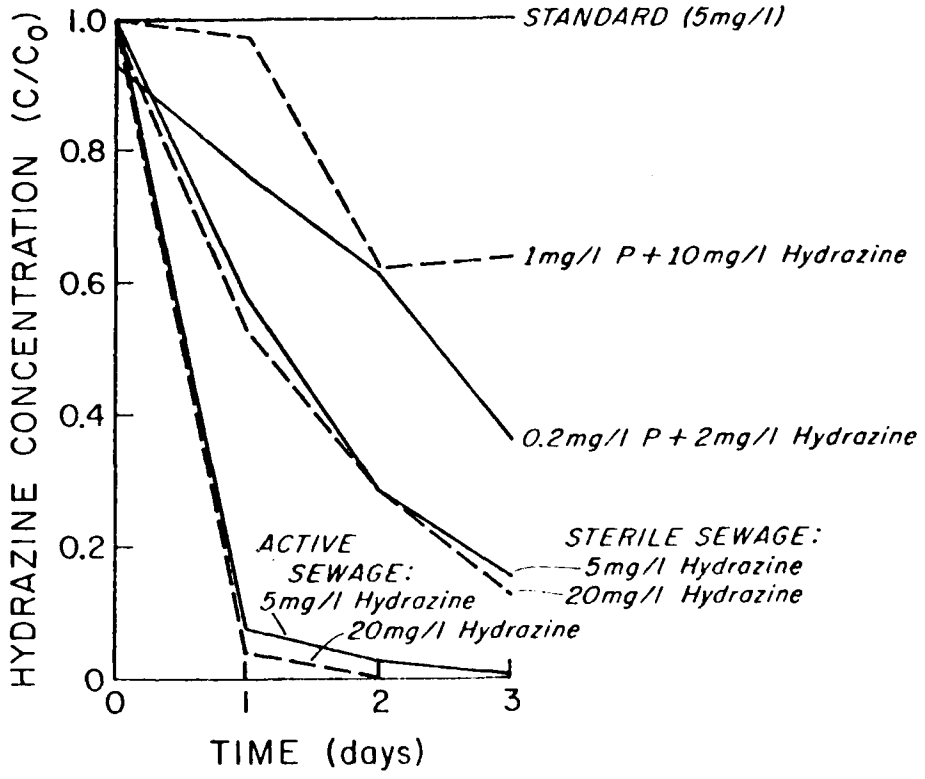

Figure 14. Degradation of Hydrazine in Various Solutions (B)

degradation was found to occur in both sterile and non-sterile sewage solutions.

A study was established using hydrazine concentrations of 5 milligrams per liter in sewage and 10 milligrams per liter in sewage which was to be autoclaved. In the latter, a final autoclaved concentration of about 5 milligrams per liter (about a 50 percent loss) was expected. All procedures and conditions were identical for both series. About 50 percent of the hydrazine in the autoclaved solutions was degraded in 48 hours (Figures 15 and 16) and was similar to that occurring in unsterile sewage (Figure 14).

For the sewage, unexpected results occurred (Figure 17). The solutions were made to yield a hydrazine concentration of 5 milligrams per liter, but less than 3 milligrams per liter was found. In addition, the hydrazine concentration appeared to increase slightly with time. The experiment was repeated with sewage solutons with an initial hydrazine dose of 10 milligrams per liter. These solutions also showed an immediate loss of hydrazine from 10 milligrams per liter to 1.7 milligrams per liter in less than 10 minutes (Figure 18).

Several explanations for these results are possible. First, the results could have been due to interferences in the hydrazine analysis from unknown compounds in the sewage. This is highly unlikely and not supported by known interferences to the DMBA method of hydrazine analysis. Second, several metals in the sewage could have resulted in rapid, catalytic oxidization of the hydrazine. This is not probable based upon known low concentrations of metals in the Corvallis sewage. Last, a biological constituent of the bacteria which is not totally destroyed from autoclaving could mediate the rapid degradation rate. This third possibility is the most likely.

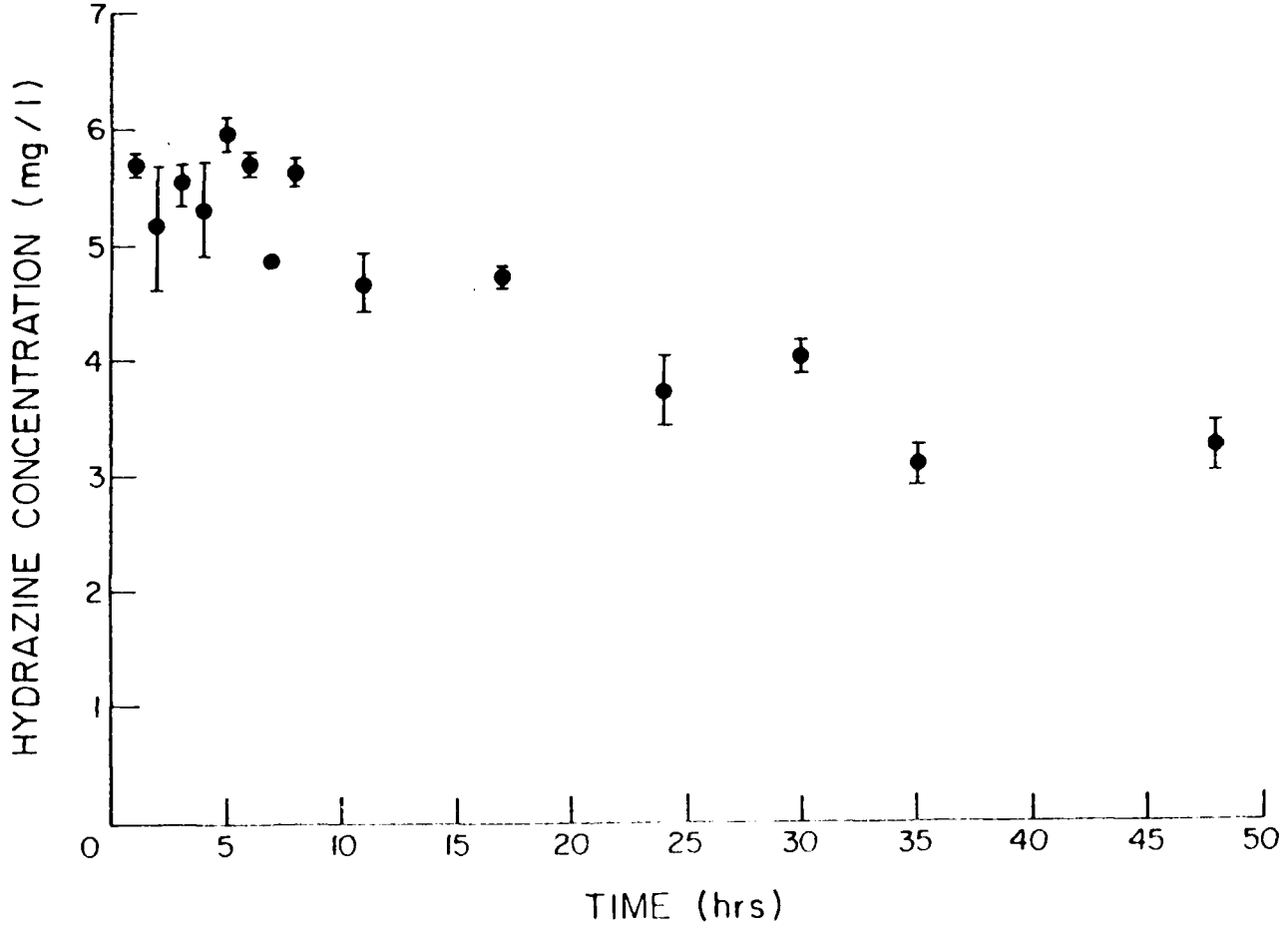

Figure 15. Degradation of Hydrazine under Sterile Conditions (◊ represents range and average) (A)

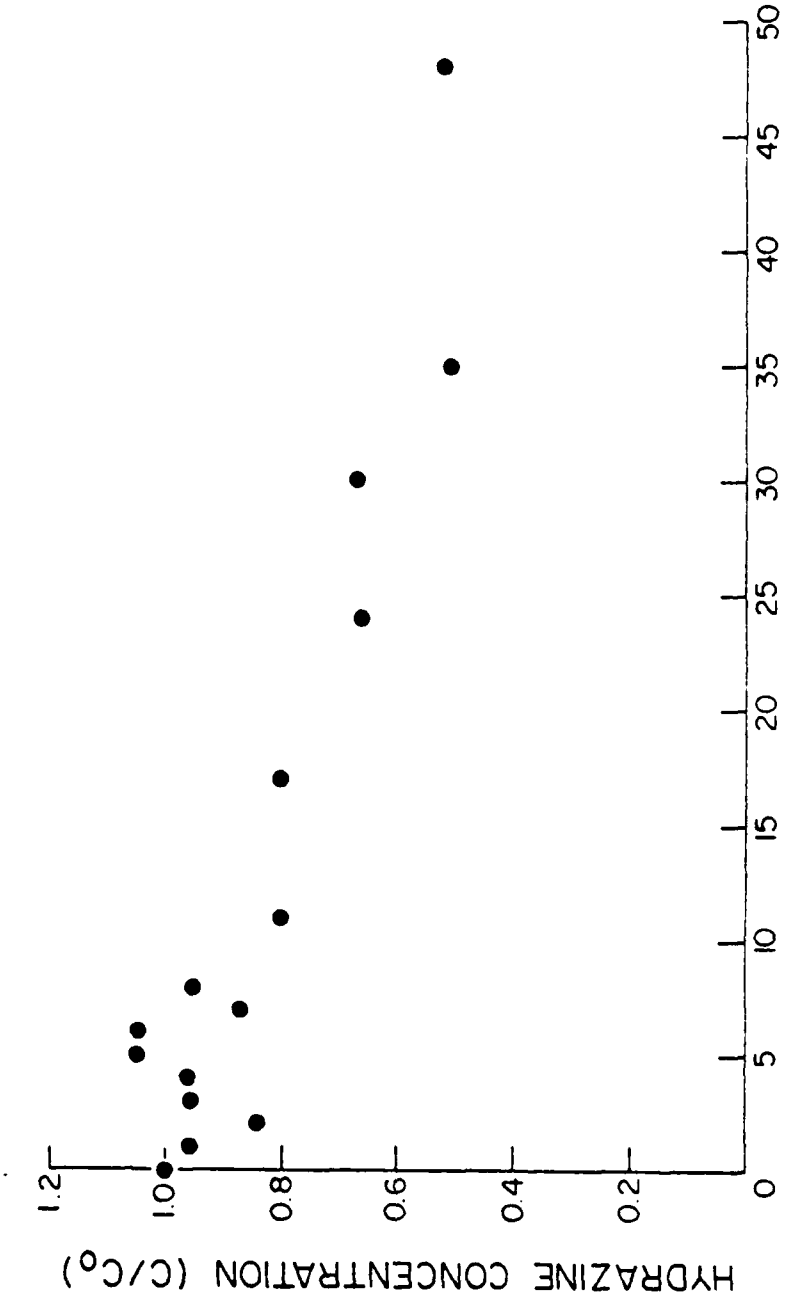

Figure 16. Degradation of Hydrazine under Sterile Conditions (B)

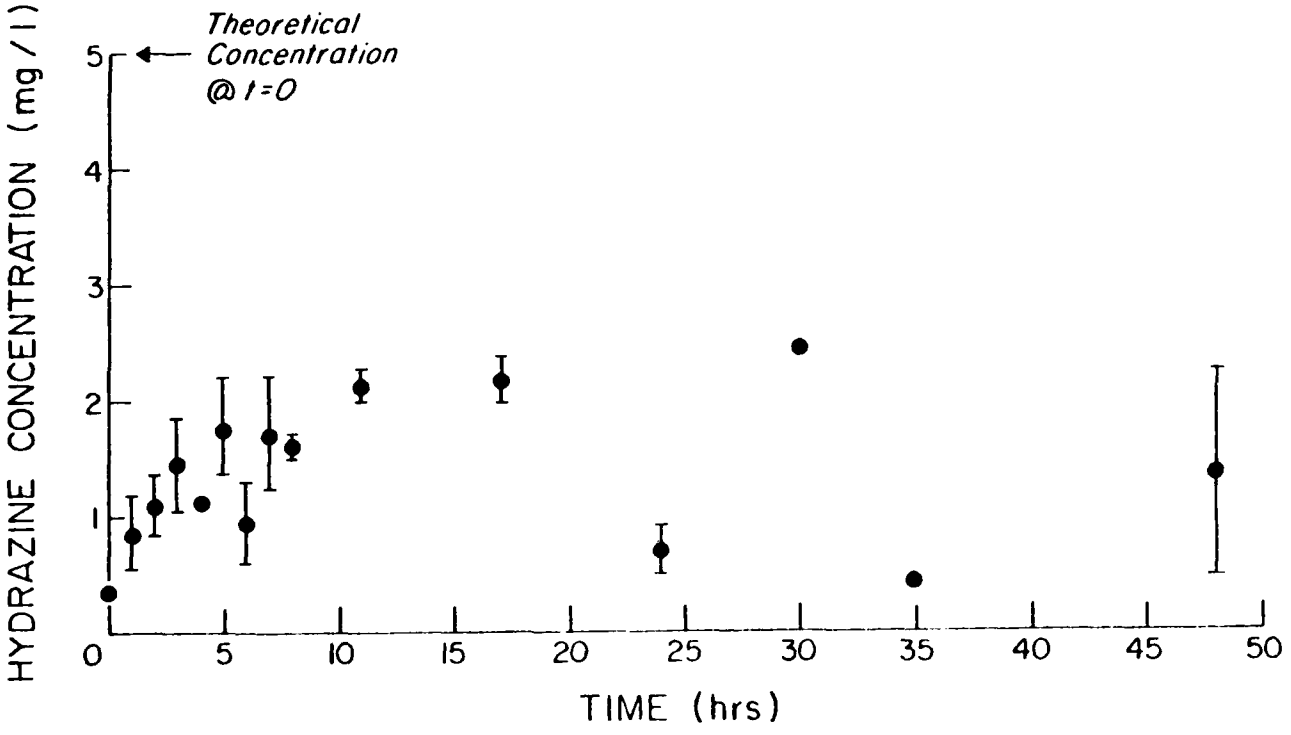

Figure 17. Degradation of Hydrazine under Non-Sterile Conditions (Φ represents range and average of experimental values) (A)

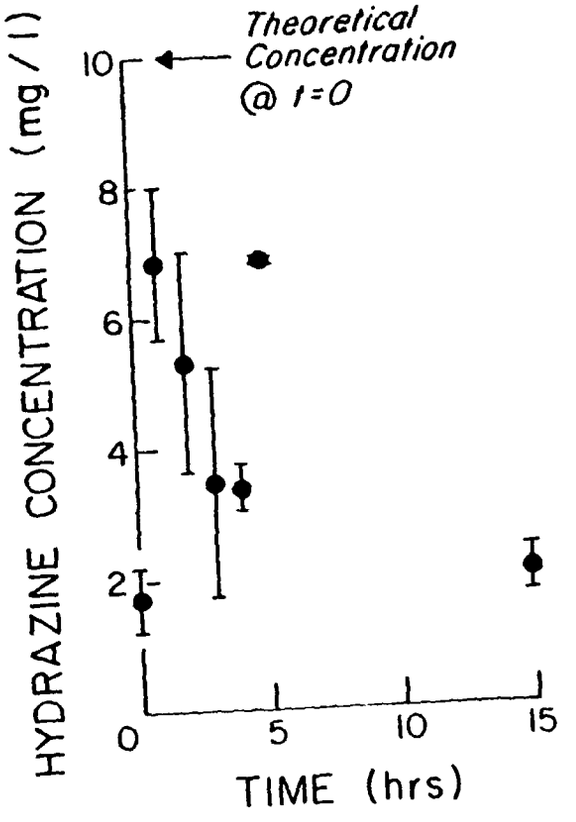

Figure 18. Degradation of Hydrazine under Non-Sterile Conditions (Φ represents the range and average of experimental values) (B)

B. COMPARATIVE CHEMICAL DEGRADATION OF HYDRAZINE, MMH, AND UDMH

All three fuels were studied under bioassay conditions with initial concentrations of 50 milligrams per liter hydrazine, 50 milligrams per liter MMH, and 100 milligrams per liter UDMH. Hydrazine fuels plus nutrients (NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and HCO_3^-) typical of beginning and midpoint bioassay concentrations were added to each flask (see Table 7).

The results of this comparative degradation of the three fuels are shown in Figures 19, 20, and 21. Hydrazine did not significantly degrade over 7 days at the unadjusted pH. Small degradation rates were observed for all solutions that did not contain NH_4^+. For the solutions containing NH_4^+, the hydrazine degradation rate was significantly greater. For MMH, the highest degradation rate occurred at a pH of 8.3. No other notable differences occurred between solutions at pH 7.0. For UDMH, the degradation rates were generally independent of pH and solution contents.

TABLE 7. CONSTITUENT ADDED FOR COMPARATIVE HYDRAZINE DEGRADATION STUDY[1]

Flask	Additive[1]	pH
1	-	unadjusted, 3.0 with H 8.3 with MM, 7.7 with, UDMH
2	-	7.0
3	15 mg/l NO_2^- - N 1 mg/l P 200 mg/l alkalinity	7.0
4	7.5 mg/l NO_2^- - N 7.5 mg/l NO_3^- - N 1 mg/l P 200 mg/l alkalinity	7.0
5	15 mg/l NH_4^+ - N 1 mg/l P 200 mg/l alkalinity	7.0
6	5 mg/l NH_4^+ - N 5 mg/l NO_2^- - N 5 mg/l NO_3^- - N 1 mg/l P 200 mg/l alkalinity	7.0

[1]Fuels added separately to different flasks with H at 50 milligrams per liter, MMH at 50 milligrams per liter, and UDMH at 100 milligrams per liter.

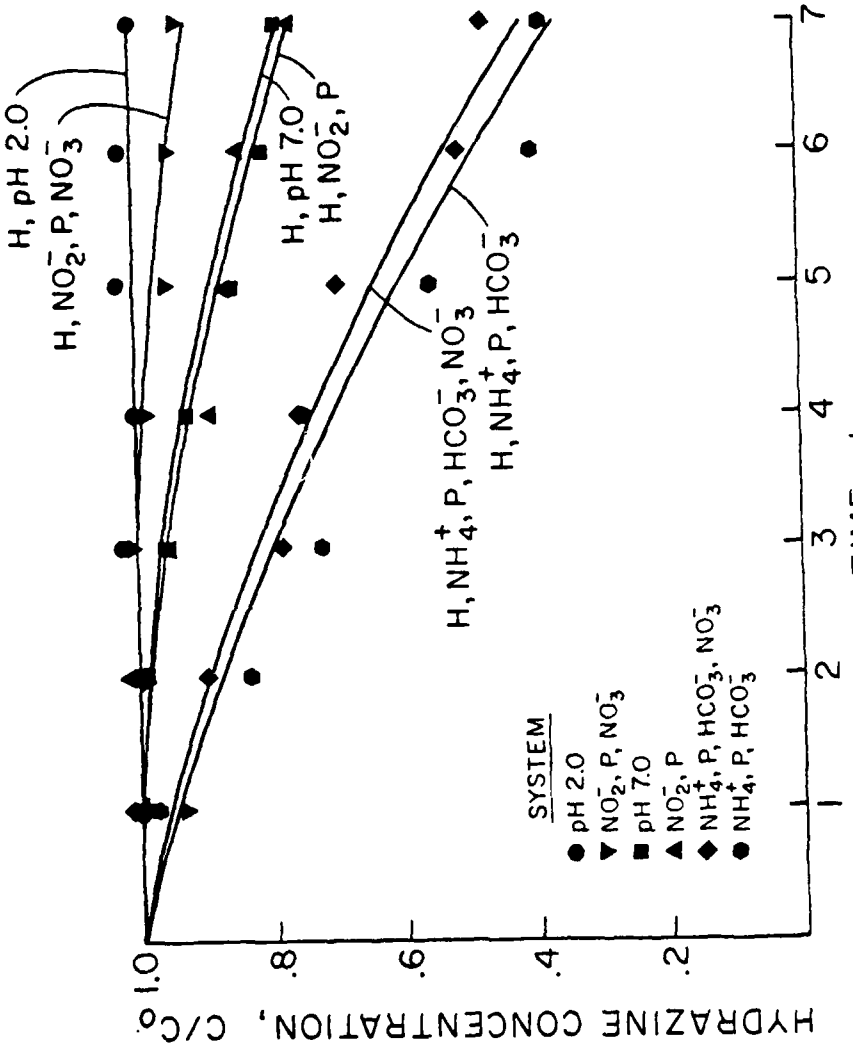

Figure 19. Hydrazine Degradation in Various Solutions

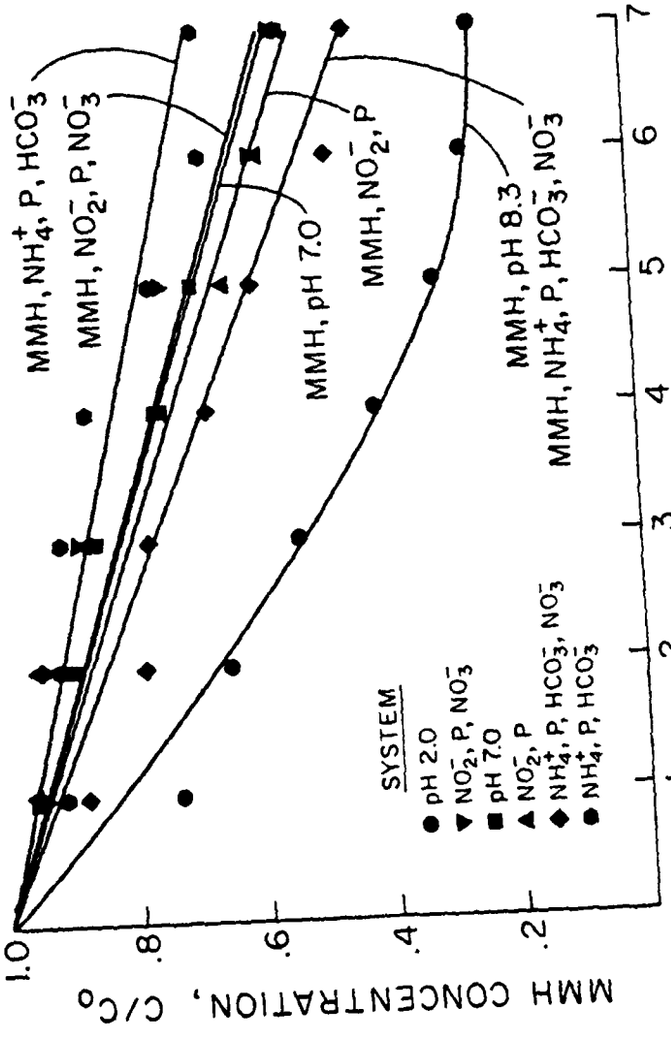

Figure 20. MMH Degradation in Various Solutions

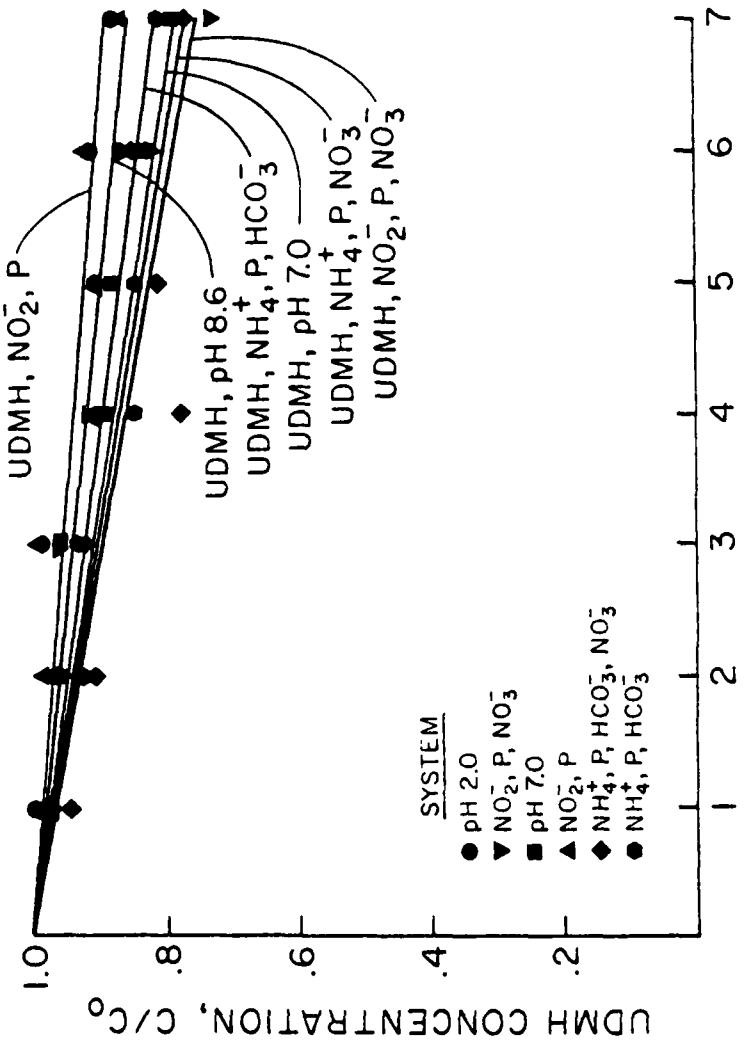

Figure 21. UDMH Degradation in Various Solutions

SECTION VIII

PRELIMINARY BIOASSAY STUDIES

Initial screening bioassays were conducted for the purpose of determining approximate ranges of toxicants. A literature review indicated that hydrazine should be toxic in concentrations from 1 to 100 milligrams per liter; this range was selected for initial screening with Nitrobacter. Nitrobacter was selected as the bioassay organism because metabolism of hydrazine was not expected and because Nitrobacter had proven an effective indicator of toxicity (Reference 28). The procedure involved placing a constant mass of Nitrobacter in a flask containing 6 milligrams per liter NO_2^- - N plus the toxicant. Nitrite concentrations were measured at 15, 60, 105, and 150 minutes or until the control flasks converted all of the nitrite to nitrate. The data for the nitrite concentrations versus time for various hydrazine concentrations are shown in Table 8 and Figure 22. These results show that 2 milligrams per liter represents a toxicity threshold with lethal doses above 10 milligrams per liter.

Approximately 4 hours after the bioassay was started, the solutions were analyzed for hydrazine (Table 9). For the non-toxic concentrations of hydrazine (0.2 through 2.0 milligrams per liter), very little hydrazine degradation occurred. However, a considerable loss of hydrazine occurred for the 10, 14, 20, and 40 milligrams per liter hydrazine solutions.

This initial screening study yielded the range (0 to 10 milligrams per liter) for conducting the Nitrobacter bioassays. The 100:1 interference ratio of NO_2^- - N to hydrazine (Section VI) dictated that the nitrite concentration should be raised to above 6 milligrams per liter. The toxic

TABLE 8. NITRITE NITROGEN CONCENTRATION VERSUS TIME FOR INITIAL NITROBACTER SCREENING STUDY

Hydrazine (mg/l)	NO_2^- (mg/l)				
	Time (min) 0	15	60	105	150
0	6.00	5.87	4.3	2.40	0.80
0	6.00	5.62	3.75	1.70	0
0	6.00	5.87	4.15	2.12	0.87
0.2	5.67	5.30	3.62	1.90	0.37
0.4	5.55	5.30	3.12	1.12	0
1.0	5.26	3.37	1.80	0.50	
2.0	5.66	5.25	3.37	1.70	0
4.0	5.55	5.37	4.62	3.25	2.12
10	5.26	5.62	5.32	5.12	5.37
14	5.08	5.70	5.30	5.00	5.00
20	4.84	5.25	4.87	4.87	4.75
40	5.55	2.5	1.65	1.10	0.87

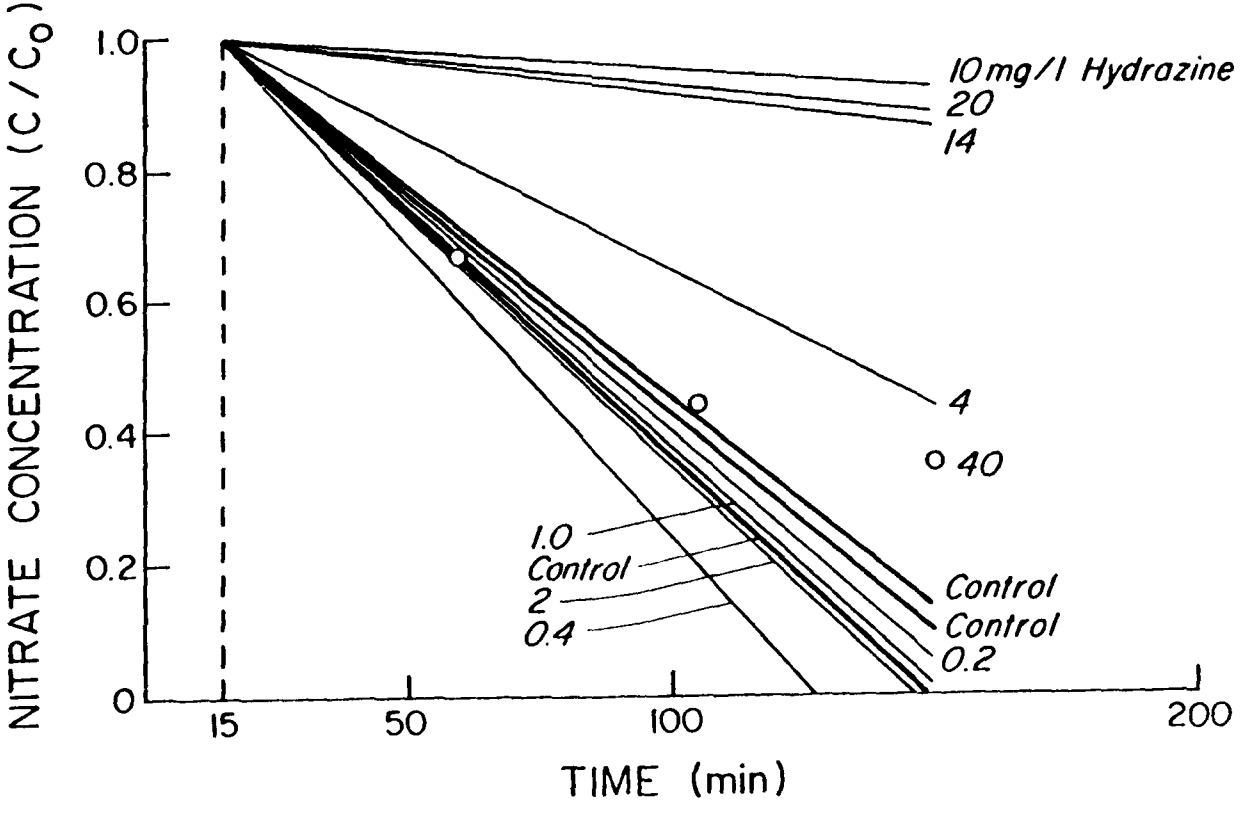

Figure 22. Nitrite Utilization for Various Hydrazine Concentrations

TABLE 9. INITIAL AND FINAL HYDRAZINE CONCENTRATION FOR *NITROBACTER* BIOASSAY

Target Conc. (mg/l)	Initial Conc. (mg/l)	Final Conc. (mg/l)	Decrease (mg/l)
0.2	0.19	0.2	-
0.4	0.37	0.3	0.07
1.0	0.87	0.75	0.12
2.0	1.89	1.6	0.29
4.0	3.7	2.95	0.75
10.0	8.8	5.75	3.05
14.0	11.9	7.0	4.9
20.0	16.1	10.4	5.7
40.0	37.0	22.8	14.2

ranges and the interferences due to hydrazine were expected to be similar for the other three bacteria populations.

SECTION IX

TOXICITY STUDIES

A. GENERAL REMARKS

The interference studies showed that a balance of NH_4^+, NO_2^-, NO_3^-, and hydrazine could not provide precise enough data to be able to specify the degradation and/or metabolism products of hydrazine. The interference study dictated that the specific ion probe method be used for NH_4^+ and NO_3^-. Consequently, a study was done to determine the compatibility of the different NH_4^+, NO_2^-, and NO_3^- analytical techniques in the presence of various concentrations of these three ions with hydrazine while maintaining a nitrogen balance.

B. NITROGEN BALANCE RESULTS IN NITROBACTER BIOASSAYS

A nitrogen balance was sought during initial Nitrobacter bioassays, and the result proved to be unsatisfactory. In these bioassays all nitrite, nitrate, and hydrazine changes were closely monitored (Table 10). All bioassays were subjected to ammonia analysis using the specific ion electrode; no ammonia was detected in any of these bioassays. It was assumed that no significant nitrogen from any source was incorporated into cellular nitrogen due to the slow growth rate of Nitrobacter. Failure of the nitrogen balance was attributed to either:

a. Low precision and/or accuracy at the concentrations tested.

b. Lack of inclusion of nitrogen gas.

After reviewing the nitrogen balance data, a study was conducted to determine the variability inherent in the analysis for hydrazine, nitrate, and nitrite at the concentrations typical of the bioassay work. Two nitrite and

TABLE 10. NITROGEN BALANCE FOR NITROBACTER BIOASSAYS

Bioassay	Initial Hydrazine (mg/l)	Final Gains or Losses (-l) ΔNO_2^- - N (mg N)	ΔN_2H_4 (mg N)	ΔNO_3^- - N (mg N)	$\frac{\Delta NO_3^- - N}{\Delta NO_2^- - N}$ (%)	$\frac{\Delta NO_3^- - N}{\Delta NO_2^- - N + \Delta N_2H_4}$ (%)
1	Controls	-0.594	-	0.250	42	42
	2	-0.511	-0.082	0.179	35	30
	5	-0.494	-0.170	0.115	23	17
	20	-0.344	-0.291	0.311	90	49
2	Controls	-0.627	-	0.391	62	62
	10	-0.377	-0.233	0.280	74	46
	25	-0.322	-0.194	0.254	79	49
	50	-0.100	-0.631	0.034	34	5
3	Controls	-0.810	-	0.534	66	66
	2	-0.710	-0.052	0.534	75	70
	20	-0.300	-0.429	0.735	245	101
	50	-0.120	-1.312	0.229	191	159
4	Controls	-1.087	-	0.498	46	46
	5	-0.855	-0.082	0.535	63	57
5	Controls	-1.366	-	1.448	106	71
	36	-0.766	-0.668	1.292	168	168
6	Controls	-2.080	-	1.970	95	77
	23	-0.912	-0.464	1.626	178	178

nitrate levels typical of initial bioassay values were selected; a 30 milligrams per liter hydrazine concentration was selected to yield about 50 percent inhibition of substrate utilization.

The data for the hydrazine concentrations with the various nitrogen additions showed that no significant interference occurred for the hydrazine analysis (Table 11). An interference in the nitrite measurement of about 5 percent was observed for solutions containing 12.5 milligrams per liter NO_2^- plus the hydrazine. The measured difference averaged 0.64 milligrams per liter NO_2^- - N less due to the presence of hydrazine. An interference in the nitrate measurement of about 15 percent was observed for solutions containing 10 milligrams per liter NO_3^- plus hydrazine. This difference was 1.84 milligrams per liter NO_3^- - N less due to the presence of hydrazine.

This analytical interference study showed that hydrazine interferes with nitrate and nitrite analysis in the range of concentration experienced in the bioassay studies. The specific ion meter was found to have significant errors between low and high nitrate levels. This resulted in a failure to achieve an accurate and consistent nitrogen balance for the bioassays. As a result, it was deemed necessary to use ^{15}N double labeled hydrazine in order to determine degradation products of hydrazine.

C. TOXICITY TO NITROBACTER

The toxicity of the hydrazine fuels to Nitrobacter was measured as the reduction of substrate utilization rate as a function of the dose of hydrazine (Figure 23). For hydrazine, the curve above about 70 milligrams per liter of hydrazine is not well defined because hydrazine at this level interferes with nitrite analysis. A concentration of 15 milligrams per liter

TABLE 11. NITROGEN BALANCE FOR INTERFERENCE OF HYDRAZINE, NO_2^- AND NO_3^-

Test	Nominal Concentration of Solution			Measured Concentrations		
	NO_2^- - N (mg/l N)	NO_3^- - N (mg/l N)	N_2H_4 (mg/l N_2H_4)	NO_2^- - N (mg/l N)	NO_3^- - N (mg/l N)	N_2H_4 (mg/l N_2H_4)
1	0	0	30	0	1.24 ±.31	32.4 ±3.2
2	0	10	0	0	12.24 ±.35	
3	0	3	0	0	4.06 ±.17	
4	12.5	0	0	13.52 ±.18	4.73 ±.41	
5	2.5	0	0	2.72 ±.15	2.05 ±.17	
6	12.5	3	30	12.88 ±.20	6.29 ±.32	32.5 ±4.1
7	2.5	10	30	2.59 ±.08	10.40 ±.51	32.9 ±3.9
8	0	0	0	0	1.18 ±.21	0

Comment: All nitrate values in tests 1 through 6 are after subtracting the nitrate concentration due to tap water alone.

hydrazine is required to produce 50-percent substrate inhibition. This compares favorably with the results of Tomlinson, Boon and Trotman (Reference 17) who found that 60 milligrams per liter produced a 75-percent inhibition level for activated sludge.

Because monomethyl hydrazine (MMH) did not interfere with the nitrite analysis, the dose-response curve at the higher doses (above 60 milligrams per liter MMH) is probably more accurate than that for the hydrazine dose-response curve (Figure 23). Comparison of this MMH dose-response curve with that for hydrazine showed that the response of Nitrobacter to both fuels is similar. This suggests that the mechanism of toxicity may be the same for both.

The response of Nitrobacter to various doses of dimethyl hydrazine (UDMH) showed a twofold order of magnitude difference as compared to the response to hydrazine or MMH (Figure 23). This suggests an entirely different toxicity mechanism.

D. TOXICITY TO NITROSOMONAS - NITROBACTER

The Nitrosomonas - Nitrobacter colony was raised as a mixed culture and, as a result, both species were present. However, the toxicity was measured for only Nitrosomonas in terms of the NH_4^+ substrate utilization as compared to controls. The response of the mixed Nitrosomonas - Nitrobacter population to hydrazine, MMH, and UDMH is given in Figure 24.

1. Hydrazine: For hydrazine, the results are plotted as a straight line as the curve of best fit. For hydrazine, a toxic response was absent at low hydrazine concentrations.

Researchers have reported that hydrazine is capable of forming an

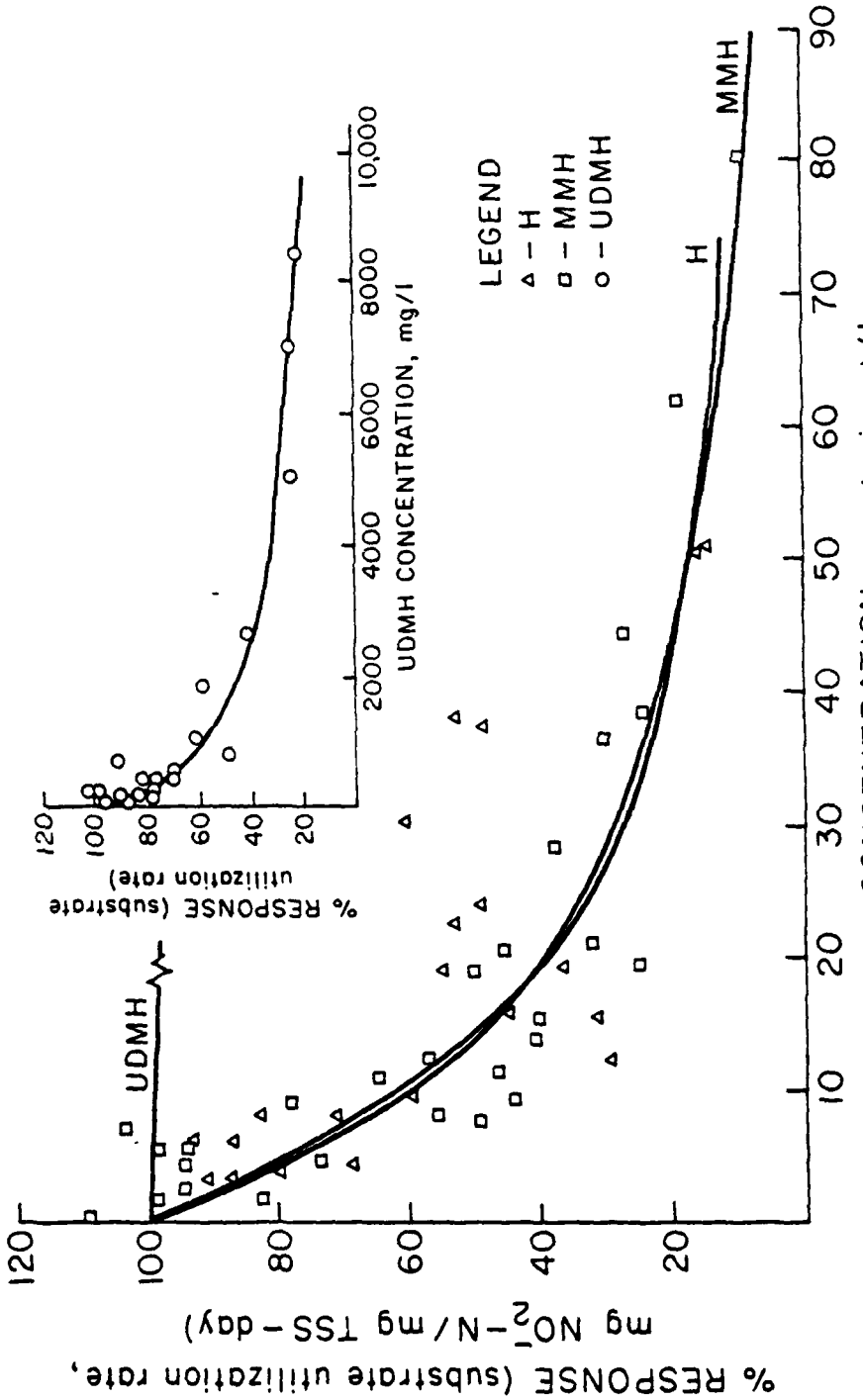

Figure 23. Toxicity of Hydrazine Fuels to *Nitrobacter*

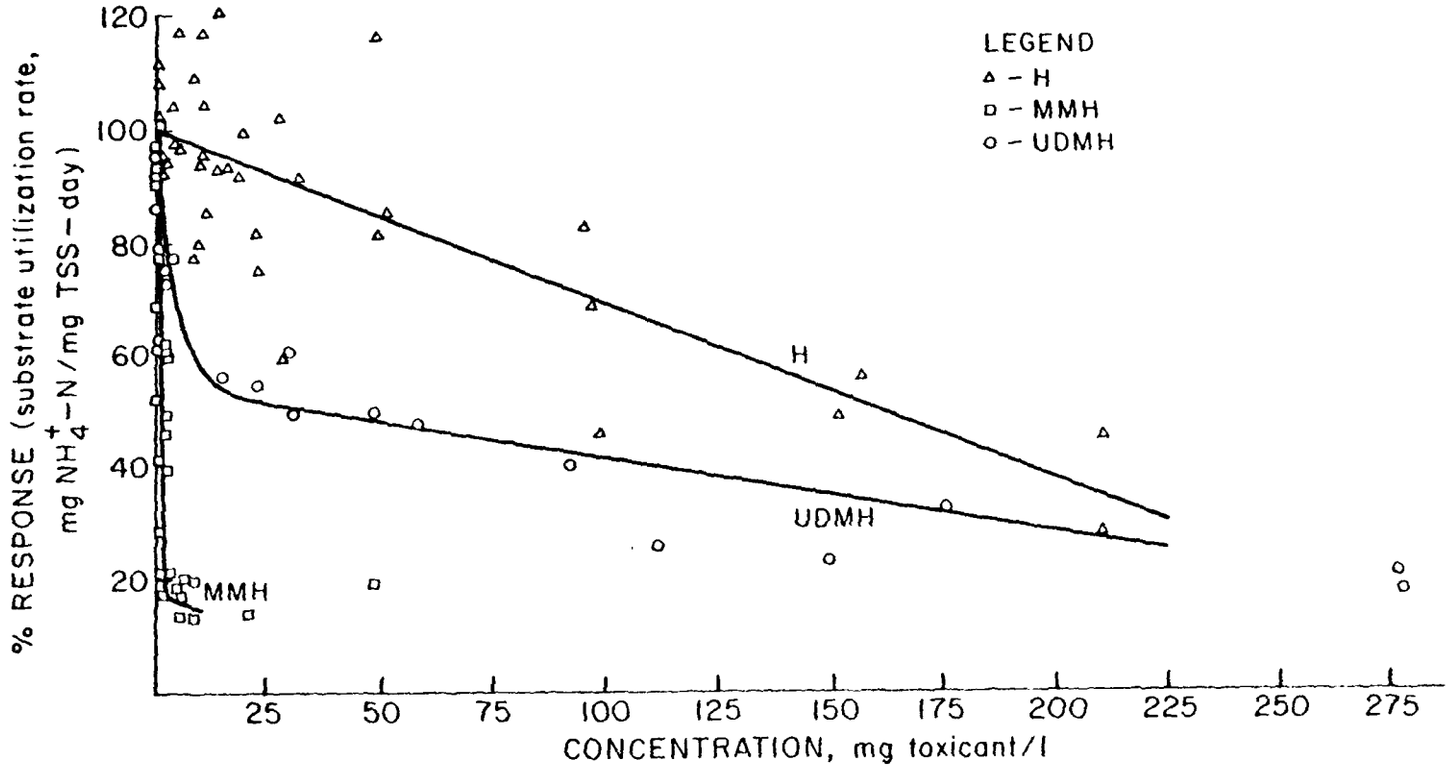

Figure 24. Toxicity of Three Hydrazine Fuels to Nitrosomonas - Nitrobacter

irreversible bond with an amine oxidase enzyme (Reference 49). To test the significance of this binding, an experiment was conducted with the Nitrosomonas - Nitrobacter culture and a hydrazine concentration of 20 milligrams per liter. The bacteria were harvested and split into equal proportions for further washing and concentration. One-half was washed with the NH_4^+ substrate and concentrated. This group was split again and introduced into the bioassay flasks. One-half of the flasks were controls and contained only the normal NH_4^+ substrate. The second half of this split was introduced into flasks with the NH_4^+ substrate and hydrazine. Next, the second half of the original harvest was washed and concentrated in tap water containing neither substrate nor hydrazine. This tap water wash eliminated residual NH_4^+ originating in the culture column. After the tap water wash, one-half of the bacteria concentrate was introduced to a hydrazine solution and incubated for 30 minutes. The scheme of this wash-concentrate-incubate routine is as follows:

SUB-GROUP	WASH	INCUBATION	BIOASSAY
1	NH_4^+	NH_4^+	NH_4^+ only
2	NH_4^+	NH_4^+	NH_4^+ + H
3	H_2O	NH_4^+	NH_4^+ only
4	H_2O	Hydrazine	NH_4^+ + H

The purpose of the water wash was to free the substrate binding sites of any NH_4^+ carried over from the culture column. If irreversible binding of the hydrazine at the substrate binding site occurred, then the group incubated with hydrazine would have been largely unable to utilize the NH_4^+ in the final bioassay. The comparison of the response of Group 2 to that

of Group 1 and of the response of Group 4 to that of Group 3 showed that no statistically significant difference existed indicating that irreversible hydrazine inhibition binding was not present for Nitrosomonas - Nitrobacter culture.

2. MMH and UDMH: Monomethyl hydrazine proved toxic to Nitrosomonas - Nitrobacter at very low concentrations (Figure 24). Fifty percent inhibition of substrate utilization occurred at less than 1 milligram per liter. The similar dose-response relationship for hydrazine and MMH as experienced with Nitrobacter was not found for Nitrosomonas. UDMH was less toxic to Nitrosomonas as the 50-percent inhibition level is about 35 milligrams per liter.

E. RESULTS OF ANAEROBIC BACTERIA TOXICITY STUDY

The batch tests of the fuels with anaerobic bacteria were run for 6 days for hydrazine, 10 days for MMH, and up to 14 days for UDMH. The latter two tests were extended to examine the possibility of acclimatization or recovery from washout. Since the batch reactors were fed daily on a fill and draw basis and each had a solids retention time of 20 days, there was a limit of about 5 to 10 days before washout of the toxicant became the dominant method of recovery. When recovery occurred between 2 and 5 days, the excess feed was rapidly digested and the daily gas production would exceed that for the controls until steady state was again reached.

1. Hydrazine: The response curves for anaerobic bacteria and hydrazine as a function of time are shown in Figure 25 and as a function of dose in Figure 26. It was necessary to crudely estimate the percent reduction of

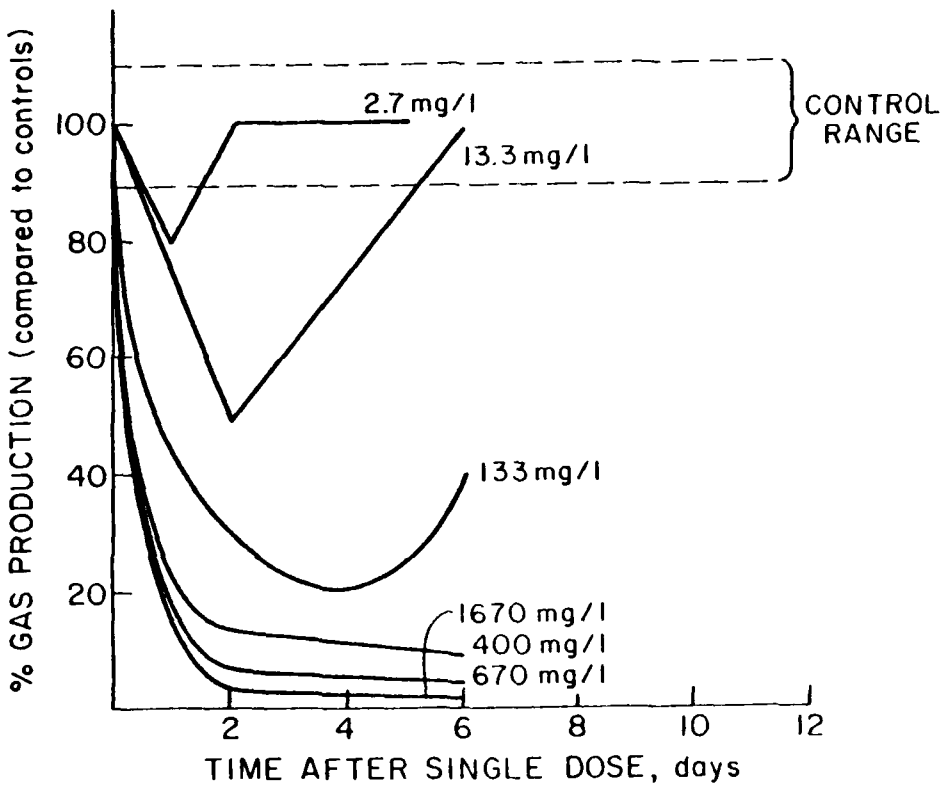

Figure 25. Gas Production of Anaerobic Bacteria Versus Time for Various Hydrazine Dosages

overall gas production versus dose so the curve in Figure 26 can only be considered to be an approximation. As such, no data points are shown. Recovery was observed for the lowest three doses of 2.7, 13.3, and 133 milligrams per liter, although for the latter, hydraulic washout of the toxicant may have been significant after about 5 days. The pH was temporarily depressed from 7.0 to 6.2 for the 2.7 and 13.3 millgrams per liter doses and continued until the third day. This probably resulted from the methane formers being more severely shocked at those concentrations than were the acid formers. No pH depression or elevation was observed at higher hydrazine doses and for all doses of MMH and UDMH.

Toxicity was measured by reduction in gas production. The toxicity for a 50-percent inhibition of gas production for hydrazine was about 100 milligrams per liter.

2. MMH and UDMH: The response of anaerobic bacteria to MMH is almost identical to that for hydrazine (Figures 26 and 27). The response to doses of 1.7 and 3.3 milligrams per liter were not significantly different from that of the controls. At 33 milligrams per liter, 50-percent reduction in gas production was experienced and then a slow recovery began to take place. No pH drop was noted at any of the doses employed. Toxicity defined as a 50-percent inhibition of gas production occurred in a range between 50 and 100 milligrams per liter.

Toxicity to UDMH occurred at a much higher concentration than for hydrazine or MMH (Figures 26 and 28). It is difficult to specify an exact dose which would yield 50-percent inhibition of gas production because a wide range of doses (332 to 5200 milligrams per liter) demonstrating toxicity within this range. However, 3000 milligrams per liter is a fair estimate of this value

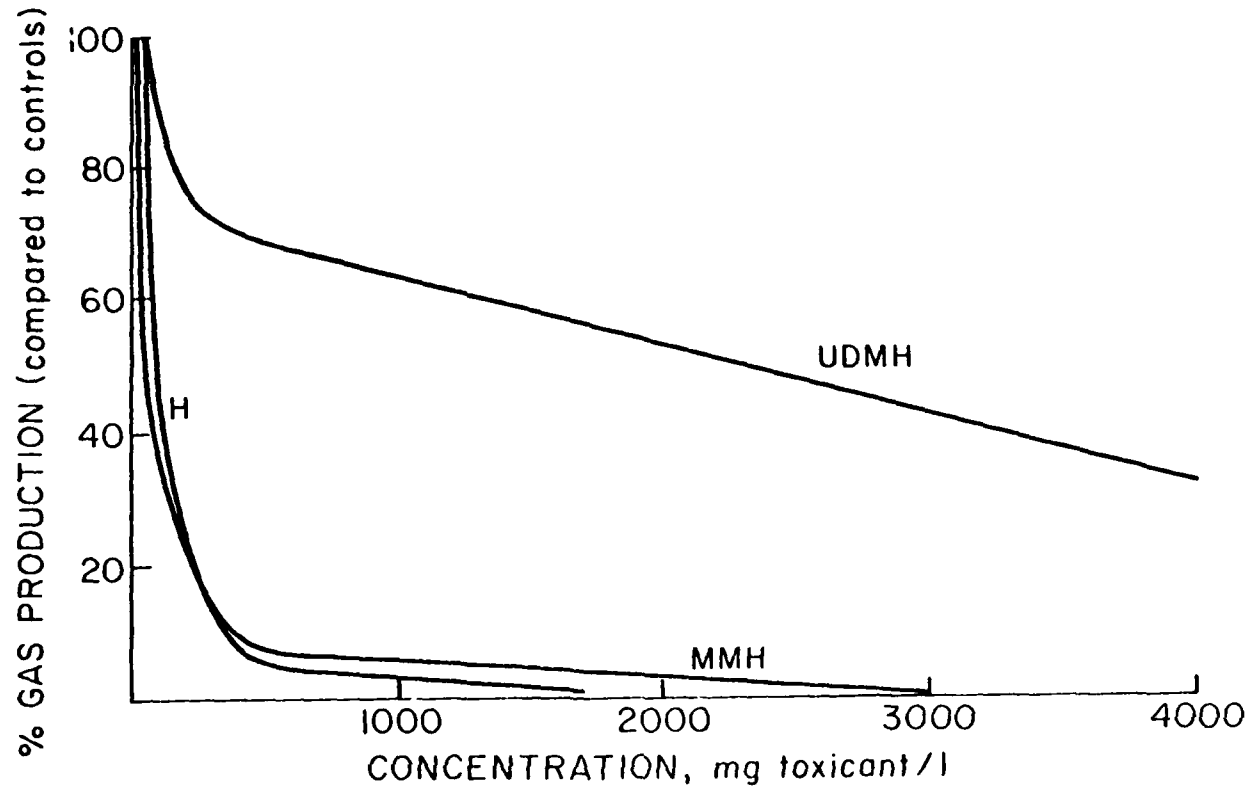

Figure 26. Toxicity of Hydrazine Fuels to Anaerobic Bacteria

Note: Curves constructed by estimating toxicant concentrations which would yield about 0, 25, 50, 75 and 100% gas production from data in Figures 25, 27 and 28.

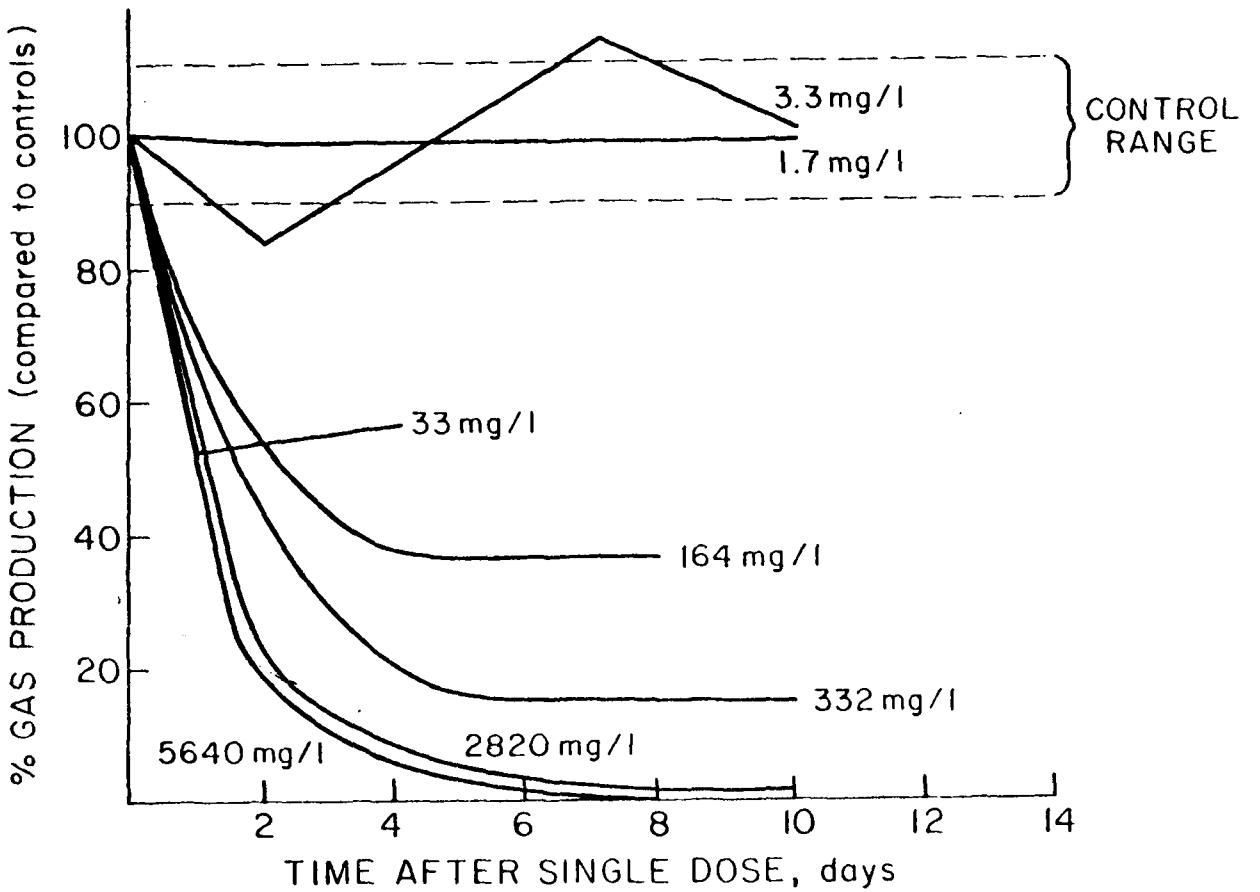

Figure 27. Gas Production of Anaerobic Bacteria Versus Time for Various Monomethyl Hydrazine Dosages

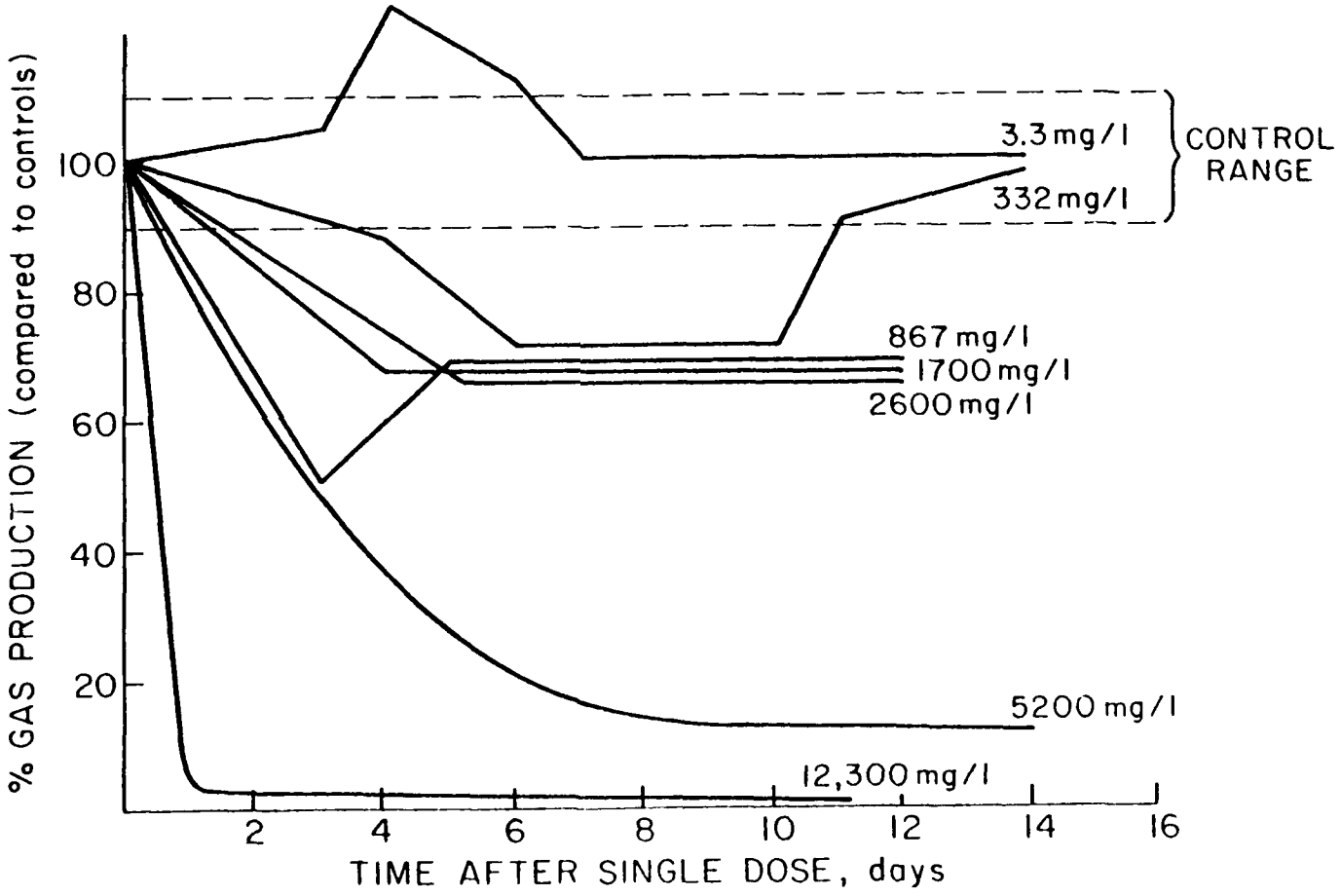

Figure 28. Gas Production of Anaerobic Bacteria Versus Time for Various Unsymmetrical Dimethyl Hydrazine Dosages

with a range of between 2600 and 5200 milligrams per liter.

F. RESULTS OF DENITRIFIER BACTERIA TOXICITY STUDY

Toxicity for the denitrifying bacteria was measured by nitrogen gas production using a Gilson respirometer. The bioassays were conducted for a period of four hours or until controls converted all of the NO_3^- substrate to nitrogen gas. It was shown that the degradation of hydrazine and MMH did not produce significant nitrogen gas to mask the nitrogen produced from nitrate reduction. The nitrogen produced from hydrazine decomposition was less than 10 percent of that produced from nitrate reduction and less for MMH. This was also attributable to the fact that only relatively low doses of both were required to produce severe toxicity. For UDMH, gas production from UDMH chemical decomposition did indeed mask the gas produced from nitrate reduction. However, this chemical reaction occurred within the first 30 minutes. Therefore, the starting point of the bioassay was delayed until this phenomena ceased.

The toxic response of the denitrifying bacteria to hydrazine is shown in Figure 29. The amount of nitrogen produced by hydrazine decomposition was accounted for by the decrease in hydrazine measured before and after the bioassays. In addition, the degree of decomposition was consistent with previously determined chemical decomposition rates for hydrazine in solutions of dead bacteria. As such, the hydrazine decomposition was not attributed to the denitrifying bacteria.

The response of the bacteria to MMH was very similar to that for hydrazine (Figure 29). Measured decreases in the MMH concentration before and after the bioassays accounted for only a small amount of gas generated over and

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BACTERIAL TOXICITY AND METABOLISM OF THREE HYDRAZINE FUELS.(U)
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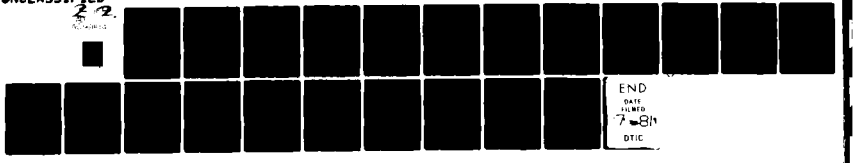

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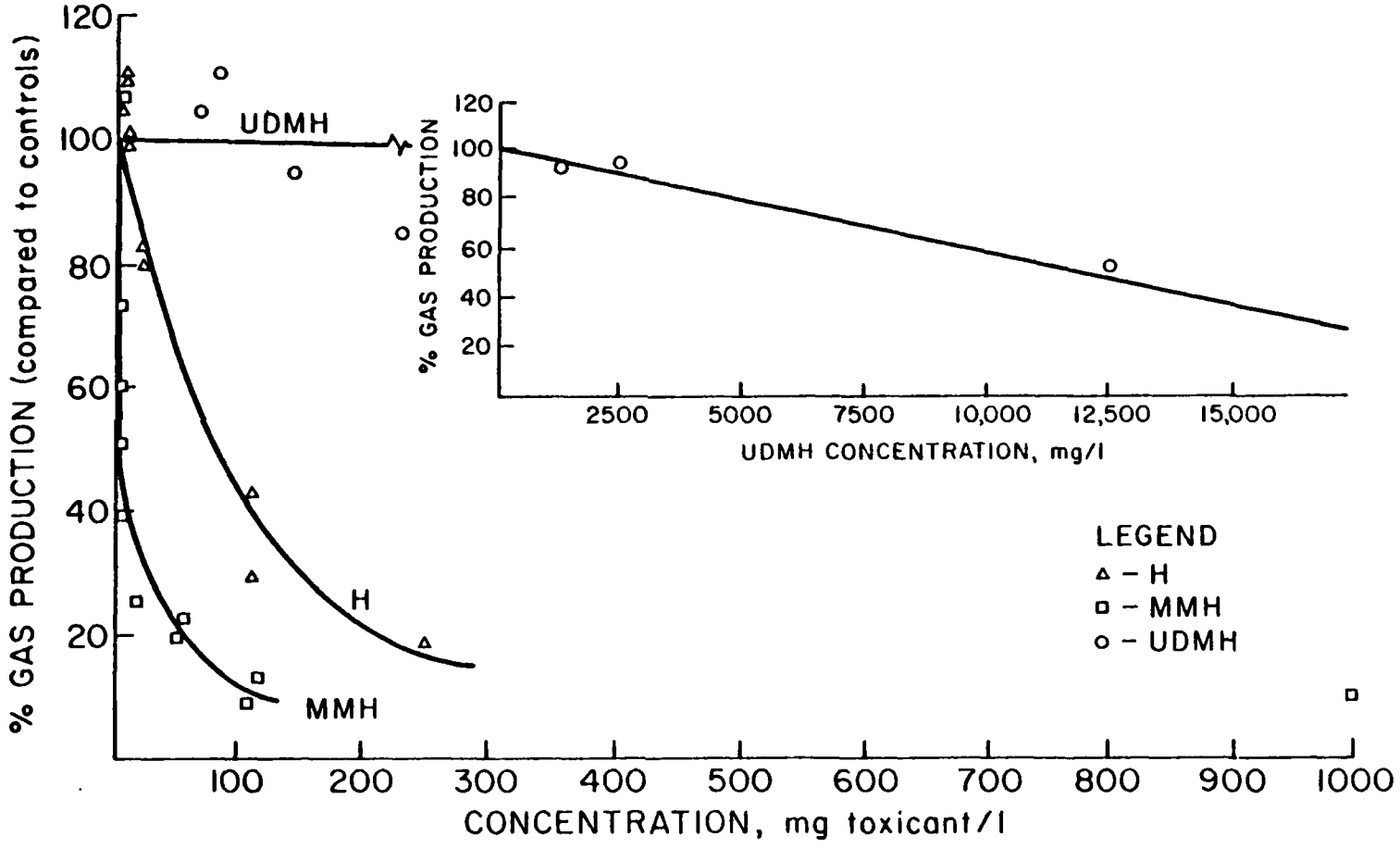

Figure 29. Toxicity of Hydrazine Fuels to Denitrifying Bacteria

above that produced by the denitrifying bacteria from nitrate reduction.

Very high doses of UDMH were required to produce significant toxicity for the denitrifying bacteria (Figure 29). Nitrogen gas from initial chemical decomposition of the hydrazine did mask nitrogen produced from nitrate reduction at UDMH doses above about 12,000 milligrams per liter. This problem was mitigated by delaying the start of the bioassay until this phase had passed and the nitrogen gas so produced was not counted. However, this problem probably produced some additional error for the dose-response curve at the higher concentrations of UDMH.

SECTION X

DEGRADATION OF HYDRAZINE

The toxicity studies demonstrated that the cultures of Nitrobacter, anaerobic bacteria and denitrifying bacteria showed a similar toxic response to the three hydrazine fuels. Although the concentrations producing 50 percent toxicity differed somewhat between cultures, the relationship between the three fuels relative to toxicity was the same for each of these three populations. In fact, hydrazine and MMH induced a similar toxic response with UDMH exhibiting much less toxicity. The Nitrosomonas population followed this same pattern in responding to MMH and UDMH, but hydrazine did not elicit a similar response. In fact, hydrazine was the least toxic of the three fuels to Nitrosomonas (Figure 27). Nitrosomonas was largely unaffected by hydrazine doses which proved toxic to other bacteria populations. From these results it was hypothesized that Nitrosomonas could metabolize hydrazine.

A. ^{15}N BIOASSAY

Two tests were required to prove this hypothesis correct. First, it must be demonstrated that hydrazine is degraded more rapidly with active as compared to dead Nitrosomonas. The procedure held all factors constant and eliminated effects of the environment (pH, temperature, light, etc.).

The second step was to use labeled hydrazine to determine the fate of the hydrazine nitrogen and support the hypothesis that the "disappearing" hydrazine was converted to nitrogen gas. These two steps together could support the hypothesis that Nitrosomonas is capable of metabolizing hydrazine to nitrogen gas at least on a short term basis.

Data for the twenty-four Nitrosomonas - Nitrobacter bioassays using ^{15}N labeled hydrazine sulphate are given in Table 12. The length of the bioassays with live bacteria averaged 5.6 hours. This was governed by the requirement that the NH_4^+ substrate not be limiting during the bioassay. The bioassays using dead bacteria (killed by raising the temperature rapidly to 90°C) were not so constrained and averaged 11.7 hours in length.

Only two bioassays using ^{15}N hydrazine in NH_4^+ substrate without bacteria were conducted because previous tests using ^{14}N hydrazine indicated that losses should be negligible. This proved to be true.

The difference in degradation rates between live and dead bacteria (0.1170 versus 0.0378 milligrams H per milligram TSS-d) was significant at the 1 percent level. This was in agreement with previous ^{14}N hydrazine bioassays on the shaker bath.

B. $^{15}N_2$ RECOVERY

The change in hydrazine concentration was determined by measuring the hydrazine before and after each bioassay along with a ^{14}N hydrazine standard as a check on the procedure, spectrophotometer, and DMBA Reagent. A period of at least 20 minutes elapsed after the bacteria were introduced into the hydrazine flask before conducting the initial hydrazine analysis. The hydrazine analysis before and after the bioassays served to determine how much hydrazine was degraded and, in turn, to calculate percent recovery.

The recovery of the hydrazine nitrogen as nitrogen gas for the bioassays is shown in Table 13. All recovered gas volumes were adjusted for volume changes in samples and standards due to temperature and atmospheric pressure.

The recovery averaged 65.7 and 64.2 percent for bioassays with live and

TABLE 12. HYDRAZINE DEGRADATION RATE FOR NITROSOMONAS - NITROBACTER USING ^{15}N LABELED HYDRAZINE

Bioassay	Length (hrs)	TSS (mg/l)	Hydrazine Lost (mg)	Hydrazine Degradation Rate (mg/hydrazine/mg TSS·d)
Live Bacteria	3.0	101.8	1.385	0.1088
	5.75	79.4	2.422	0.1273
	5.5	88.3	1.964	0.0971
	4.0	90	3.773	0.2515
	4.0	86.5	3.454	0.2396
	3.0	88.5	1.987	0.1796
	4.25	120.3	1.709	0.0803
	5.53	146.0	1.584	0.0471
	4.95	84.3	1.238	0.0712
	7.5	96.4	1.728	0.0574
	9.0	86.3	1.673	0.0517
	6.7	71.5	2.522	0.1263
	6.8	109.9	3.545	0.1138
	9.0	123.9	3.999	0.0861
	AVG 5.64	AVG 98.1		AVG 0.1170
Dead Bacteria	3.0	92.0	0.907	0.0789
	4.12	107.4	0.684	0.0371
	11.0	108.9	1.419	0.0284
	13.0	93.2	1.219	0.0241
	18.0	78.2	1.556	0.0265
	13.2	102.4	1.453	0.0258
	14.25	64.7	1.557	0.0405
	17.4	63.0	1.871	0.0409
	AVG 11.75	AVG 88.7		AVG 0.0378
Tap Water	6.58	-0-	-0-	-0-
	14.25	-0-	-0-	-0-
	AVG 10.4	AVG -0-		AVG -0-

TABLE 13. RECOVERY RATE FOR ^{15}N BIOASSAYS

Bioassay Type	% Recovery	Remarks
Live Bacteria		
1	79.41	
2	72.97	
3	-	Leak at Mass Spectrometer
4	87.22	
5	52.61	
6	68.55	
7	54.76	
8	-	Data lost in computer
9	44.02	
10	82.60	
11	63.40	
12	-	Data lost in computer
13	59.77	
14	57.71	
	$\bar{X}$ = 65.73 ± 13.64	
Killed Bacteria		
1	71.78	
2	64.74	
3	47.84	
4	84.49	
5	45.51	
6	47.48	
7	48.89	
8	102.91	
	$\bar{X}$ = 64.21 ± 21.02	
NH_4 in Tap Water		
1	-0-	No Hydrazine degraded
2	-0-	No Hydrazine degraded

dead bacteria, respectively. The probable reason for the less than full recovery rate was leakage of $^{14}N_2$ into the system. This raised the total volume of gas collected to 40 milliliters ml which is the maximum the mass spectrophotometer can accept. Above 40 milliliters, positive pressure will break the sampler-mass spectrophotometer connection and release some gas. The major contaminant in the samples was nitrogen ($^{14}N_2$) gas which leaked in any one of over 100 joints and connectors. The leak rate was constant over time, but specific leak sources could not be pin-pointed. Although O_2 also leaked into the system, the pyrogallic acid and secondary O_2 trap removed much of this contaminant.

The recovery rate was large enough to support the hypothesis that at least the major decomposition product of hydrazine was indeed nitrogen gas. To search for other possible gaseous or liquid by-products using ^{15}N labeled hydrazine was beyond the scope and capability of this research. Modification to procedures and equipment could have been made to convert NH_4^+ in the bioassay flask to N_2 and to capture this gas, but did not appear to justify the expense. The method for such a procedure has been described by Ross and Martin (Reference 50) and Porter and O'Deen (Reference 51). A search for other nitrogen compounds would require still more complicated procedures.

SECTION XI

ACCLIMATIZATION TO HYDRAZINE

A single study was initiated to determine if it was possible for bacteria to acclimatize to hydrazine. Depending upon the length of time allowed for the development and upon the concentration of hydrazine employed, the mechanism of adaption would either be acclimatization or mutation.

The study involved subjecting a culture of Nitrosomonas - Nitrobacter to continuous feed of 40 milligrams per liter of hydrazine. A small colony of Nitrosomonas - Nitrobacter was started using the effluent containing NH_4^+ and some bacteria from the larger Nitrosomonas - Nitrobacter colony. The 40-milligrams per liter level was chosen since previous studies had shown that Nitrosomonas would experience about a 15-percent reduction in substrate utilization rate and Nitrobacter would experience about 80-percent reduction in substrate utilization rate.

The substrate utilization rate for the colony began a uniform drop to zero in 10 days after continuous hydrazine addition. No subsequent recovery was noticed during the next 25 days. At this point, bioassays were conducted to see if the culture could metabolize NH_4^+ with and without the hydrazine. No substrate utilization occurred. No further attempts of acclimatization were made because of concern for hydrazine exposure of other personnel.

SECTION XII

DISCUSSION

The twofold purpose of this research was to determine the toxicity of three hydrazine fuels to four populations of bacteria and to determine the fate of hydrazine in a microbial environment. The four bacteria populations were selected because of their role in the nitrogen cycle and because there was a possibility that one of these four groups might be able to metabolize hydrazine.

A. TOXICITY

The toxicity studies indicated the toxic levels of the three fuels to each of the four bacteria populations. These results are in general agreement with results reported in the literature. However, the response of Nitrosomonas to hydrazine is unlike those of the other three populations as seen by comparing Figure 24 with Figures 23, 26, and 29. Hydrazine, while very toxic to the other three populations including Nitrobacter, does not induce a similar response in Nitrosomonas. This supports the hypotheses that hydrazine may be metabolized by Nitrosomonas.

There are several possible biochemical sites for inhibition by hydrazine. In very high concentrations, hydrazine reacts with proteins to cleave C-terminal amino acids and release them as acylhydrazines and amines (Reference 52).

At least one site for hydrazine inhibition of Nitrosomonas is known and reported extensively in the literature (References 19,22,27,53, and 54). Intact cells will oxidize ammonia to nitrite with hydroxylamine (NH_2OH) as an intermediate in the process. The conversion of hydroxylamine to nitrite

is rapid and thought to provide the free energy for the first step. Hydrazine in the concentration range of 3.2 to 32 milligrams per liter reportedly inhibits the process and hydroxylamine accumulates. Hydroxylamine itself is toxic at 28 milligrams per liter (Reference 55).

Considerable work has also been done with various portions of cell free constituents of Nitrosomonas and the results are somewhat clouded. Ritchie and Nicholas (Reference 56), and Anderson (Reference 53) have shown that in addition to hydroxylamine (NH_2OH), other intermediates or side reaction metabolites of the oxidation of ammonia to nitrite include the nitroxyl form (NOH), hyponitrite ($N_2O_2H_2$), nitric oxide (NO) and nitrous oxide (N_2O). Both oxidase and reductase activity has been demonstrated under aerobic and anaerobic conditions. Other researchers (Reference 57) have implicated the electron transport system of Nitrosomonas as susceptible to inhibition by hydrazine.

In relation to the metabolism of hydrazine by Nitrosomonas, Anderson (Reference 19) has indicated that hydrazine probably competes with hydroxylamine and is biochemically dehydrogenated. However, Nicholas and Jones (Reference 22) suggest that the inhibition of nitrite formation is due to competition with hydroxylamine for a common acceptor such as cytochrome C.

The results of this study have shown that hydrazine is inhibitory and toxic to Nitrobacter, Nitrosomonas, denitrifiers, and anaerobic bacteria as reported by others (References 32 and 33). The mechanisms for such reactions are unknown. If the electron transport system is the site of inhibition as suggested for Nitrosomonas, then it would be reasonable to expect the same system to be affected by hydrazine in the other populations.

The degradation of hydrazine in the presence of Nitrosomonas is significantly larger than in the presence of dead Nitrosomonas or as compared to

chemical degradation. This increased degradation rate was not found when hydrazine was exposed to the other three bacteria populations. These results further support the hypotheses that Nitrosomonas can metabolize hydrazine.

Hydrazine oxidation to N_2 or to other possible intermediate metabolites is an energy yielding process which could supply Nitrosomonas with energy for respiration and growth. In addition, hydrazine is chemically similar to ammonia. The oxidation of NH_4^+ and hydrazine by two electron transfer steps would yield NO_3^- and N_2, respectively. Hydrazine and ammonia are both protonated as $N_2H_5^+$ and NH_4^+ at neutral pH [pk_A for hydrazine is 7.9 (Reference 58]). This suggests that hydrazine and NH_4^+ are similar as substrates for Nitrosomonas and that the final metabolic product of hydrazine should be nitrogen gas.

The study using ^{15}N labeled hydrazine clearly showed that hydrazine was rapidly degraded by Nitrosomonas and the primary final degradation product of hydrazine is nitrogen gas, not NH_4^+, NO_2^-, or NO_3^-.

B. ACCLIMATIZATION

The inability of Nitrosomonas to acclimate to hydrazine was expected. The N-N bonded compounds in nature are rare, while those manufactured number in the hundreds. The ability of various organisms to form the N-N is widely distributed and, as such, metabolism would be expected. In general, however, the naturally occurring N-N compounds are toxic (Reference 59).

The search for a bacteria capable of utilizing the N-N compounds for energy and synthesis has not been fruitful. LaRue and Child (Reference 60) screened 26 compounds containing the N-N bond (Table 14), and 25 of them failed to serve as nitrogen source for soil-isolated bacteria. Pseudomonas was able to utilize the cyclic hydrazine derivative 1,4,5,6 - tetrahydro - 6 - oxo - 3 - pyridazine carboxylic acid (PCA) as sole nitrogen source,

TABLE 14. N-N BONDED COMPOUNDS USED AS POTENTIAL MICROBIAL NITROGEN SOURCE (30)

Methylhydrazine	3,5-Pyrazole dicarboxylic acid
sym-Dimethyl hydrazine	N-pyrazolyl propionic acid
n-Propyl hydrazine	N-pyrazolyl acetic acid
N,N'-dicarbethoxy hydrazine	N-(beta hydroxyethyl) pyrazole
Methyl hydrazine carboxylate	3-Carbethoxy-pyrazole-5-one
N,N'-diacetyl hydrazine	3-Methyl-2-pyrazoline-5-one
Malonic hydrazide	4-Methyl-2-pyrazoline-5-one
β-hydroxy-DL-butyric hydrazide	3,4-Dimethyl-pyrazol-5-one
Cyclopropane carboxylic hydrazide	3-Pyrazolidinone
4-Amino-1,2,4-triazole	3,6-Dihydroxy-4-methyl pyridazine (citraconic hydrazide)
N-amino pyrrolidine	1,2-Dihydro-3,6-pyridazine dione (maleic hydrazide)
Pyrazole	1,2-Dihydro-4-methyl-3,6-pyridazinedione
3,5-Dimethyl pyrazole	*1,4,5,6-Tetrahydro-6-oxo-3-pyridazinecarboxylic acid (PCA)

*The only compound supporting growth of a Pseudomonas sp.

but was unable to metabolize the other 25 compounds tested.

LaRue (Reference 59) reported no literature references indicating any of the N-N compounds that could be used as sole nitrogen source. Bulen (Reference 61) showed that hydrazine could not serve as nitrogen source for nitrogen fixing bacteria. Even finding an organism or plant containing a hydrazine derivative has proven difficult. Helweg (Reference 62) showed that although the plant growth regulator maleic hydrazide is degraded in soil, the specific organism or organisms responsible for this phenomenon could not be isolated.

SECTION XIII

ENGINEERING SIGNIFICANCE

This research was undertaken to provide information on the fate and effect of hydrazines in an aquatic environment. The increased use and transportation of hydrazine throughout the world has increased the possibility of an accidental release of the hydrazines to the environment. Because of the size of some of the rail and truck transport containers, the volumes from a spill could easily be large enough to cause significant environmental damage. At these Air Force and NASA facilities routinely handling the hydrazine, disposal of small amount of the fuels, especially hydrazine, poses a difficult problem.

As the hydrazines are released into the environment, chemical and biological degradation of the hydrazines will occur. The hydrazines will be evaporated to some degree and decomposed to nitrogen gas and other products by chemical reactions catalyzed by heat and certain metals. The hydrazines that enter the aquatic environment will also begin to decompose, but at a very slow rate as shown in this research. Decomposition will not be rapid enough to reduce hydrazine to below toxic levels.

Except for Nitrosomonas, hydrazine and MMH are highly toxic to the bacteria populations and UDMH is moderately toxic. These bacteria populations are all integral parts of the aquatic environment. Once destroyed in a spill situation, recovery could not be expected until all traces of the fuels are removed. Even though Nitrosomonas appears able to metabolize hydrazine the prevalence of this bacteria in a natural aquatic environment is not adequate to significantly mitigate damage from a hydrazine spill.

Since Nitrosomonas can metabolize hydrazine and do represent a relatively large portion of the total bacteria population in a biological waste water treatment plant, this suggests the possibility of using conventional biological treatment processes for routine treatment of hydrazine wastes. However, such a system is probably not feasible since:

a. Nitrosomonas were not able to mutate or survive during a long term exposure to a hydrazine concentration of only 40 milligrams per liter.

b. Nitrobacter are very sensitive to low levels of hydrazine and would not be able to continue its role in the conversion of NO_2^- to NO_3^- in nitrogen cycle.

c. Other heterotrophic bacteria as in activated sludge are affected at levels of hydrazine well below the levels toxic to Nitrosomonas ( ).

The results of this study would suggest extreme caution in disposal of waste hydrazine fuels to biological waste treatment plants. Conventional neutralization procedures using chlorination followed by dechlorination is probably still preferred as a more feasible treatment method.

SECTION XIV

CONCLUSIONS

Based upon the results of this study, the following conclusions are made.

1. Concentrations of hydrazine to reduce metabolism by 50 percent were for Nitrobacter, Nitrosomonas, denitrifying bacteria, and anaerobic bacteria 15, 165, 100, and 100 milligrams per liter, respectively.
2. Concentrations of monomethyl hydrazine to reduce metabolism by 50 percent were for Nitrobacter, Nitrosomonas, denitrifying bacteria, and anaerobic bacteria were 15, <1, 10, and 75 milligrams per liter, respectively.
3. Concentrations of unsymmetrical dimethyl hydrazine to reduce metabolism by 50 percent were for Nitrobacter, Nitrosomonas, denitrifying bacteria, and anaerobic bacteria were 1800, 35, 12,500, and 2300 milligrams per liter, respectively.
4. Nitrosomonas can metabolize hydrazine to nitrogen gas, but apparently cannot metabolize MMH or UDMH.
5. The use of conventional biological treatment processes is not recommended for treatment of these three fuels. Even at low concentrations, serious toxicity can be expected.
6. Spills of these three fuels into the aquatic environment can be expected to seriously disrupt natural bacterial populations.

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